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(54) Title: PEPTIDE EPITOPES RECOGNIZED BY ANTIGEN SPECIFIC CD8+ T LYMPHOCYTES

(57) Abstract: The invention provides methods for identifying and validating epitopes that are bound to class I MHC molecules and activate $CD8^+$ T cells involved in the pathogenesis of or protection from diseases, e.g., cancers. The invention includes peptide epitopes derived from the CEA polypeptide by such methods, and methods of therapeutic use of these epitopes against diseases such as cancers.

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PEPTIDE EPITOPES RECOGNIZED BY ANTIGEN SPECIFIC CD8$^+$ T LYMPHOCYTES

Field of the Invention

The invention relates to the identification of naturally processed and presented HLA class I restricted peptides and their use as therapeutics and prophylactics.

Background of the Invention

After proteolytic processing of intact protein antigens by antigen presenting cells (APCs), class I Major Histocompatibility Complex (MHC) molecules on the APCs bind short antigenic peptides (epitopes) derived from the antigens, presenting the bound peptides to CD8$^+$ T lymphocytes [Germain and Margulies (1993), Ann. Rev. Immunol. 11:403-450]. Class I MHC genes and the molecules they encode are highly variable among individuals, and differences among the class I MHC molecules determine which peptides are selected for presentation as T cell epitopes. Identification of the naturally processed peptide fragments of a polypeptide of interest that are presented by class I MHC molecules of a subject can be useful for developing peptides that regulate immune response to the polypeptide in that subject.

A growing body of evidence suggests that CD8$^+$ T lymphocytes are important in the immune response to tumor cells. CD8$^+$ T lymphocytes recognize peptide epitopes in the context of HLA class I molecules that are expressed on the surface of almost all nucleated cells. Following intracellular processing of endogenously synthesized tumor

antigens, antigen-derived peptide epitopes bind to class I HLA molecules in the endoplasmic reticulum. The resulting complex is transported to the cell surface. $CD8^+$ T lymphocytes recognize the peptide-HLA class I complex, which results in the destruction of the cell bearing the HLA-peptide complex directly by the $CD8^+$ T lymphocytes and/or via the activation of non-destructive mechanisms, *eg.*, activation of lymphokines such as tumor necrosis factor -α (TNF -α) or interferon- γ (IFN γ) which enhance the immune response and facilitate the destruction of the tumor cell.

A fundamental challenge in the development of an efficacious tumor immunomodulatory preparation is immune suppression or tolerance that can occur. There is therefore a need to establish immunomodulatory embodiments that elicit immune responses of sufficient breadth and vigor to prevent progression and/or clear the tumor. Persons skilled in the art can also refer to U.S. Patent Application 60/232,185 (incorporated herein by reference) which describes identification and use of $CD4^+$ T lymphocytes CEA peptide epitopes.

The epitope approach employed in the present invention represents a solution to this challenge, in that it allows the incorporation of $CD8^+$ T lymphocytes epitopes, from discrete regions of a target tumor-associated antigen (*TAA*) in a single immunomodulatory composition. Such a composition can simultaneously target multiple dominant and subdominant epitopes and thereby be used to achieve effective immunization in a diverse population.

Carcinoembryonic antigen (CEA) was first described by Gold and Freedman (1965), J. Exp. Med. 121:439-462. CEA is

a highly glycosylated, 180,000-dalton protein that is expressed on most gastrointestinal carcinomas, including colon, rectal, pancreatic and gastric (Muraro *et al.*, *Cancer Res.* 45:5769-5780, 1985) as well as 50% of breast (Steward *et al.*, *Cancer (Phila)* 33:1246-1252, 1974) and 70% of non-small cell lung carcinomas (Vincent et *al.*, *J. Thorac. Cardiovasc. Surg.* 66:320-328, 1978). It is also found on some normal, endodermally derived tissues, though in much lower concentrations. The abnormally high expression on cancer cells makes CEA an important target for immunotherapy. CEA cell surface expression can be detected using the monoclonal antibody (mAb) Col-1, and CEA can be identified in total cellular protein by western blot analysis using the same mAb. An MHC class I (HLA-A2)-restricted $CD8^+$ T lymphocytes response against a processed CEA epitope has been described in patients with metastatic disease [Tsang et al. (1995), J. Natl. Cancer. Inst. 87:982-990]. This MHC class I-restricted epitope was identified by taking peripheral blood lymphocytes from patients after immunization with a recombinant vaccinia virus encoding CEA and screening the T cell response against synthetic 9- to 11-mer CEA peptides. $CD8^+$ T lymphocytes from three patients were shown to have specificity for a class I-restricted CEA epitope in an assay using autologous Epstein Barr Virus (EBV)-transformed B cells pulsed with a 9 amino acid synthetic peptide derived from CEA.

SUMMARY OF THE INVENTION

The invention features methods for identifying peptide epitopes that activate $CD8^+$ T lymphocyte responses involved

in the initiation, promotion, or exacerbation of certain diseases.

The invention is based on the discovery that one can identify HLA class I-restricted epitopes naturally produced in tumor cell lines. These peptide epitopes can be incorporated into a drug delivery system and used to elicit an epitope-specific $CD8^+$ T cell response in an appropriate mammalian subject. Where the epitope is derived from a tumor antigen, such a response promotes the migration of $CD8^+$ T lymphocytes to the vicinity of tumor cells expressing the tumor antigen and specific targeting of the tumor cells by the $CD8^+$ T lymphocytes.

More specifically, the invention features a method of identifying a class I MHC-binding fragment of a polypeptide. The method involves: (a) isolating from tumor cell lines a class I MHC molecule bound to a peptide, the peptide being a class I MHC-binding fragment of the polypeptide; (b) eluting the peptide from the class I MHC molecule; (c) identifying the peptide as a fragment of the polypeptide; (d) chemical verification of the peptide by HPLC elution time mapping and LC/MS/MS fragmentation comparison to a synthetic homologue; (e) measuring the binding affinity of the peptide to the specific class I MHC molecule; and (e) immunological validation by stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a synthetic peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks, followed by specific recognition of the target tumor cells by the peptide specific generated $CD8^+$ T cells. The polypeptide can have the sequence of a tumor antigen. The tumor cells can be any class I MHC-expressing mammalian

cell, e.g., gastric carcinoma, colorectal adenocarcinoma, breast carcinoma, lung carcinoma, myeloma, glioma, or lymphoma, and the mammal from which it is derived can be a human. The class I MHC molecule can be a HLA-A molecule, or a HLA-B molecule, or a HLA-C molecule. Such a HLA-A molecule can be encoded by a A*0101, A*0201, A*0301, A*1101, or A*2402 gene.

The invention provides an isolated peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNDQSL (SEQ ID NO:2), where the aspartic acid at position P7 is a post translationally deamidated asparagine residue at P7 of (SEQ ID NO:1). Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNGQSL (SEQ ID NO:3), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLSGANLNL (SEQ ID NO:4), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGVLVGVALI (SEQ ID NO:5), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer

than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGV (SEQ ID NO:6), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence IMIGVLVGV (SEQ ID NO:7), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also embraced by the invention are altered peptide ligands (APL), which are class I MHC-binding variant peptides in which 1 to 6 amino acid residues are different from the corresponding residues of the wild-type class I MHC-binding peptide, but which still bind to the same class I MHC molecules as the wild-type peptides, corresponding to SEQ ID NOS:1-8, as are methods of therapy and prophylaxis involving the use of APL. APL have the ability to elicit different patterns of cytokine production in T cells than do their parent wild-type peptides. Thus, for example, while a wild-type peptide presented by a MHC molecule may induce production of Th1 cytokines, an APL derived from it and presented by the same MHC molecule may elicit Th2 or other immunoregulatory cytokines. Alternatively, the wild-type peptide may stimulate the production of Th2 cytokines while a corresponding APL elicits production of Th1 cytokines.

Another aspect of the invention is a method of activating T cell reactivity in a mammal. The method

involves: (a) providing (i) at least one peptide, the sequence of which consists of the sequence of a naturally processed fragment of CEA, the peptide being capable of binding to a class I MHC molecule of the mammal and of eliciting a $CD8^+$ T cell response, or (ii) a nucleic acid encoding a polypeptide that is (1) the peptide, (2) the peptide plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and (b) administering the peptide or nucleic acid to the mammal. The peptide can include or be YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The above described method of identifying a class I MHC-binding fragment of a polypeptide can include the additional steps of: (e) providing $CD8^+$ lymphocytes from a mammal having a condition suspected of being associated with presentation of the peptide by the class I MHC molecule, the APCs of the mammal bearing the class I MHC molecule;(f) providing a population of APCs that bear the class I MHC molecule with the peptide bound thereto; (g) contacting the population of APCs of (f) with the $CD8^+$ lymphocytes of (e); and (h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC-bound peptide, as an indication that presentation of the peptide to $CD8^+$ T lymphocytes is associated with the condition. The presentation can be associated with a pathological response of $CD8^+$ T lymphocytes or a protective response of $CD8^+$ T lymphocytes.

Another aspect of the invention is a method of diagnosis that involves: (a) providing a $CD8^+$ lymphocyte from an individual suspected of having or being susceptible to cancer; (b) providing an APC which bears on its surface a class I MHC molecule of an allele identical to one expressed by the individual, the class I MHC molecule being bound to a CEA peptide; (c) contacting the APC with the $CD8^+$ lymphocyte; and (d) determining whether the $CD8^+$ lymphocyte recognizes the class I MHC-bound peptide, as an indication that the individual has or is susceptible to cancer. The peptide can include or be the amino acid sequence YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The invention also features a method of treating a subject suspected of having or being susceptible to cancer. The method involves administering a peptide or isolated nucleic acid to the subject. The peptide or peptide encoded by isolated nucleic acid can be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNDQSL (SEQ ID NO:2), where the aspartic acid at position P7 is a post translationally deamidated asparagine residue at P7 of (SEQ ID NO:1). Alternatively, the peptide could be fewer than

17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNGQSL (SEQ ID NO:3), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLSGANLNL (SEQ ID NO:4), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGVLVGVALI (SEQ ID NO:5), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGV (SEQ ID NO:6), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence IMIGVLVGV (SEQ ID NO:7), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The invention also provides a method of identifying a reagent for diagnosing cancer. The method involves: (a) providing a test reagent that can be a Fab fragment, a monoclonal antibody (mAb), or a single chain Fv (scFv) fragment; (b) providing a complex that contains a class I MHC molecule bound to a peptide that includes or is

YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends; and (c) testing whether the test reagent binds to the complex. The class I MHC molecule can a HLA-A2 molecule encoded by a A*0201 gene.

Also embraced by the invention is a method of diagnosis. The method involves:(a) providing a test cell from a mammalian subject; (b) providing a reagent that binds to a CEA peptide fragment bound to a class I MHC molecule; (c) contacting the test cell with the reagent; and (d) detecting binding of the reagent to the test cell as an indication that the test cell is a cancer cell.

The invention also features a method of cancer therapy that involves: (a) providing a composition that includes a reagent that can be a Fab fragment, a mAb, or a scFv fragment, the reagent being able to recognize a naturally processed CEA peptide bound to a MHC class I molecule and being linked to an agent that can be a chemotherapeutic compound, a radioactive isotope or a toxin; and (b) administering the composition to a subject suspected of having or being susceptible to a cancer characterized by expression of CEA.

Another aspect of the invention is a method of identifying a class I MHC-binding fragment of a tumour antigen. The method involves: (a) providing a mammalian cell that contains a class I MHC molecule and the tumour antigen; (b) isolating from the cell the class I MHC molecule bound to a peptide, the peptide being a class I MHC binding fragment of the tumour antigen;(c) eluting the

peptide from the class I MHC molecule; and (d) identifying the amino acid sequence of the peptide. The method can involve the additional steps of: (e) providing $CD8^+$ lymphocytes from a mammal having a cancer suspected of being associated with presentation of the peptide by the class I MHC molecule, the cells of the mammal bearing the class I MHC molecule; (f) providing a population of cells that bear the class I MHC molecule with the peptide bound thereto; (g) contacting the population of cells of (f) with the $CD8^+$ lymphocytes of (e); and (h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC bound peptide, as an indication that presentation of the peptide to $CD8^+$ lymphocytes is associated with the cancer.

The invention also provides an isolated nucleic acid that contains a nucleotide sequence that encodes a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. The peptide encoded by the nucleic acid thus can include or be the amino acid sequence YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also featured by the invention is a vector containing nucleic acid that encodes one or more of the peptides that are defined above. In the vector the nucleotide sequence can be operatively linked to a transcriptional regulatory element. Also included in the invention is a cell (e.g., a mammalian, an insect, a bacterial, a yeast, or a fungal cell) containing any of the vectors of the invention.

An "isolated" peptide of the invention is a peptide which either has no naturally-occurring counterpart (e.g., an APL), or has been separated or purified from components which naturally accompany it, e.g., in tissues such as pancreas, liver, spleen, ovary, testis, muscle, joint tissue, neural tissue, gastrointestinal tissue, or body fluids such as blood, serum, or urine. The peptide is considered "isolated" when it is at least 70%, by dry weight, free from the proteins and peptides with which it is naturally associated. Preferably, a preparation of a peptide of the invention is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, the peptide of the invention. Since a peptide that is chemically synthesized is, by its nature, separated from the components that naturally accompany it, the synthetic peptide is, by definition, "isolated."

An isolated peptide of the invention can be obtained, for example, by extraction from a natural source (e.g., from human tissues or bodily fluids); by expression of a recombinant nucleic acid encoding the peptide; or by chemical synthesis. A peptide that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will be separated from components that naturally accompany it. The extent of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, mass analysis, or HPLC analysis.

An "isolated nucleic acid " means nucleic acid free of the genes that flank the gene of interest (e.g., the gene encoding CEA) in the genome of the organism in which the

gene of interest naturally occurs. The term therefore includes a recombinant nucleic acid incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote. It also includes a separate molecule such as: a cDNA where the corresponding genomic DNA has introns and therefore a different sequence; a genomic fragment; a fragment produced by polymerase chain reaction (PCR); a restriction fragment; a nucleic acid encoding a non-naturally occurring protein, fusion protein, or fragment of a given protein; or a nucleic acid which is a degenerate variant of a naturally occurring nucleic acid. In addition, it includes a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Also included is a recombinant nucleic acid that includes a nucleic acid sequence that encodes any of the peptides with SEQ ID NOS:1-8.

As used herein, "protection from a mammalian disease" means prevention of onset of a mammalian disease or lessening the severity of a disease existing in a mammal. "Prevention" can include a delay of onset, as well as a partial or complete block in progress of the disease or disease symptoms.

As used herein, "a naturally-processed peptide fragment" is a peptide fragment produced by proteolytic degradation of a protein in an antigen presenting cell of a mammal. As used herein, a "tumor antigen" is a molecule (e.g., a protein molecule) that is expressed by a tumor cell. Such a molecule can differ (e.g., by one or more amino acid residues where the molecule is a protein) from, or it can be identical to, its counterpart expressed in

normal cells. The tumor antigen is preferably not expressed by normal cells. Alternatively, it is expressed at a higher level (e.g., a two-fold, three-fold, five-fold, ten-fold, 20-fold, 40-fold, 100-fold, 500-fold, 1,000-fold, 5,000-fold, or 15,000-fold higher level) in a tumor cell than in the tumor cell's normal counterpart. Examples of tumor antigens include, without limitation, CEA, prostate specific antigen (PSA), MAGE (melanoma antigen) 1-4, 6, 11-12, A10, and C1, MUC (mucin) (e.g., MUC-1, MUC-2, etc.), tyrosinase, MART (melanoma antigen), Pmel 17(gp100), GnT-V intron V sequence (N-acetylglucoaminyltransferase V intron V sequence), Prostate Ca psm, PRAME (melanoma antigen), β-catenin, MUM-1-B (melanoma ubiquitous mutated gene product), GAGE (melanoma antigen) 1, BAGE (melanoma antigen) 2-10, c-ERB2 (Her2/neu), EBNA (Epstein-Barr Virus nuclear antigen) 1-6, gp75/TRP 1, human papilloma virus (HPV) E6 and E7, p53, lung resistance protein (LRP) Bc1-2, and Ki-67, RAGE, LAGE-1, CAG-3, DAM, NY-ESO- 1, CDK4, BRCA2, NY-LU- 1, NY-LU-7, NY-LU- 12, CASP8, RAS, KIAA-2-5, SCCs, p53, p73, CEA, Her 2/neu, Melan-A, TRP2, kallikrein, PAP, PSA, PT 1-1, B-catenin, Telomerase, FAK, cyclin D1 protein, NOEY2, EGF-R, SART- 1, CAPB, HPVE7, p 15, Folate receptor CDC27, PAGE- 1, and PAGE-4. Recognition of such a peptide by $CD8^+$ T cells of a mammal (e.g., a human patient) is indicative of the existence, or future onset, of cancer in the mammal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred

methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. Unless otherwise indicated, these materials and methods are illustrative only and are not intended to be limiting. All publications, patent applications, patents and other references mentioned herein are incorporated by reference.

Other features and advantages of the invention, e.g., methods of identifying peptides that activate $CD8^+$ T lymphocyte responses, will be apparent from the following description, from the drawings and from the claims.

This invention applies our knowledge of the mechanisms by which antigen is recognized by T cells, for example, to develop epitope-based immunomodulatory preparations directed towards TAAs. More specifically, this application communicates our discovery of specific epitope pharmaceutical compositions and methods of use in the prevention and treatment of cancer.

Upon development of appropriate technology, the use of epitope-based immunomodulatory preparations has several advantages over current vaccines, particularly when compared to the use of whole antigens in vaccine compositions. For example, immunosuppressive epitopes that may be present in whole antigens can be avoided with the use of epitope-based immunomodulatory preparations. Such immunosuppressive epitopes may, e.g., correspond to immunodominant epitopes in whole antigens, which may be avoided by selecting peptide epitopes from non-dominant regions *(see, e.g., Disis et al., J. Immunol. 156:3151-3158, 1996)*.

An additional advantage of an epitope-based immunomodulatory preparations approach is the ability to combine selected epitopes and further, to modify the composition of the epitopes, achieving, for example, enhanced immunogenicity. Accordingly, the immune response can be modulated, as appropriate, for the target disease. Similar engineering of the response is not possible with traditional approaches.

Another major benefit of epitope-based immunomodulatory preparations is their safety. The possible pathological side effects caused by infectious agents or whole protein antigens, which might have their own intrinsic biological activity, is eliminated.

An epitope-based immunomodulatory preparation also provides the ability to direct and focus an immune response to multiple selected antigens from the same pathogen (a "pathogen" may be an infectious agent or a tumor-associated molecule). Thus, patient-by-patient variability in the immune response to a particular pathogen may be alleviated by inclusion of epitopes from multiple antigens from the pathogen in an immunomodulatory composition.

Furthermore, an epitope-based anti-tumor immunomodulatory preparation also provides the opportunity to combine epitopes derived from multiple tumor-associated molecules. This capability can therefore address the problem of tumor-to-tumor variability that arises when developing a broadly targeted anti-tumor immunomodulatory preparation for a given tumor type and can also reduce the likelihood of tumor escape due to antigen loss. For example, a breast cancer tumor in one patient may express a target TAA that differs from a breast cancer tumor in

another patient. Epitopes derived from multiple TAAs can be included in a polyepitope immunomodulatory preparation that will target both breast cancer tumors.

Brief Description of the Drawings

Fig. 1 is a MS/MS Spectrum of naturally processed YLWWVNNQSL (SEQ ID NO:1). Spectral labeling in Figs. 1-7 use the nomenclature described by Roepstorff and Fohlman (1988), Biomed. Mass. Spectrom. 11:601, and Biemann (1990), Meth. Enzymol. 193:866-867.

Fig. 2 is a MS/MS spectrum of naturally processed YLWWVNDQSL (SEQ ID NO:2)

Fig. 3 is a MS/MS spectrum of naturally processed YLWWVNGQSL (SEQ ID NO:3).

Fig. 4 is a MS/MS spectrum of naturally processed YLSGANLNL (SEQ ID NO:4). There were a number of background responses (marked by *) that were derived from a precursor ion that co-eluted chromatographically with YLSGANLNL (SEQ ID NO:4) and was closely related in m/z. These Such background could be only partially resolved chromatographically from YLSGANLNL (SEQ ID NO:4) but this did not prevent the identification of YLSGANLNL (SEQ ID NO:4) from the resultant composite mass spectrum.

Fig. 5 is a MS/MS spectrum of naturally processed ATVGIMIGV (SEQ ID NO:6).There were a number of background responses (marked by *) that were derived from a precursor ion that co-eluted chromatographically with ATVGIMIGV (SEQ ID NO:6) and was closely related in m/z. These Such background could be only partially resolved chromatographically from ATVGIMIGV (SEQ ID NO:6) but this

did not prevent the identification of ATVGIMIGV (SEQ ID NO:6) from the resultant composite mass spectrum.

Fig. 6 is a MS/MS spectrum of naturally processed IMIGVLVGV (SEQ ID NO:7).

Fig. 7 is a MS/MS spectrum of naturally processed GVLVGVALI (SEQ ID NO:8).

Fig. 8 is the primary sequence of CEA with the SEQ ID NO 1-5 highlighted. The c-terminal 16 amino acids contains SEQ ID NO. 5-8.

DETAILED DESCRIPTION

The peptide epitopes and corresponding nucleic acid compositions of the present invention are useful for stimulating an immune response to a TAA by stimulating the production of $CD8^+$ T lymphocyte responses. The peptide epitopes, which are derived directly or indirectly from native TAA protein amino acid sequences, are able to bind to HLA molecules and stimulate an immune response to the TAA. The complete sequence of the TAA proteins to be analyzed can be obtained from GenBank. Peptide epitopes and analogs thereof can also be readily determined from sequence information that may subsequently be discovered for heretofore unknown variants of particular TAAs, as will be clear from the disclosure provided below.

The present invention is based on the novel discovery that peptide epitopes from tumor cells can be isolated, identified and used to stimulate $CD8^+$ T cells capable of recognizing tumor cells in mammals (e.g. disease tissue in human patients). The TAA are degraded by proteolytic enzymes into peptide fragments. If any of these peptide fragments has, by virtue of its length and sequence, the ability to bind to one of the class I MHC molecules

expressed by the cell, it will do so in the endoplasmic reticulum. The resulting peptide-class I MHC molecular complex is then transported to the cell's cell membrane, where it becomes available for interaction with $CD8^+$ T cells bearing antigen-specific receptors that specifically recognize that particular peptide-class I MHC complex. By eluting peptides from class I MHC molecules isolated from these cells, a set of naturally processed peptides derived from the TAA, as well as from other polypeptides of intracellular or extracellular origin, is obtained. The peptides, which are specific to the particular types (isotypes and alleles) of class I MHC molecules expressed by the APC, are then chemically separated and their amino acid sequences determined. By comparison of the peptide amino acid sequences to the sequence of the TAA, it is possible to identify those which are derived from the TAA. Thus, the present invention includes a method of identifying peptide fragments that are naturally processed by APC and have intrinsic binding affinity for the relevant class I MHC molecule. The method can be invaluable for identifying peptides derived from a polypeptide suspected of being an antigen that activates $CD8^+$ T cells involved in either (a) the pathogenesis (pathology) of a disease, especially one in which susceptibility or protection is known to be associated with expression of a particular type of class I MHC molecule, or (b) prevention or reduction of the symptoms of a disease, especially one in which protection or a reduction in severity is associated with expression of a particular type of class I MHC molecule.

The described method ensures that the peptides identified are those that both (i) are naturally processed

in vivo by the cell, and (ii) become associated, in the cell, with the relevant class I MHC molecules.

Furthermore, the present method controls for class I MHC type, an important aspect essential to link any given peptide to a particular $CD8^+$ T cell-mediated disease in a given individual but especially important in disorders in which class I MHC type determines disease susceptibility or resistance.

Any naturally processed peptide with a sequence that corresponds to a fragment of the TAA, and which binds to a class I MHC molecule associated with the disease of interest, could be a peptide that activates $CD8^+$ T cells that exacerbate the disease or mediate immunity to it. To obtain confirmatory evidence of this possibility, test $CD8^+$ T cells from subjects expressing the relevant class I MHC molecules can be assayed for responsiveness to a peptide identified in accordance with the invention. A significant response of the test $CD8^+$ T cells and no or little response of the control $CD8^+$ T cells would indicate that the relevant peptide is involved either in the disease process (pathology of the disease) or in immunity to the disease. The cellular response phase of the method is designated "Epitope Verification" ("EV").

By applying the methods of the invention to the tumor antigen CEA, CEA-derived peptides were identified as epitopes that could be involved in the pathogenesis of cancer in human patients expressing the HLA-A2 class I MHC allele. Based on their amino acid sequences, these peptides fall into 5 domains. A consensus peptide corresponding to the binding region of each domain can be synthesized and tested for its ability to activate $CD8^+$

T cells from HLA-A2-expressing cancer patients or donors and recognizing tumor cells expressing both HLA-A2 and CEA.

Various strategies can be utilized to evaluate immunogenicity, including:

1) Evaluation of primary T cell cultures from normal individuals (*see, e.g.*, Wentworth, P. A. *et al.*, *Mol. Immunol.* 32:603, 1995; Celis, E. *et al.*, *Proc. Mad. Acad Sci.* USA 91:2105, 1994; Tsai, V. *et al.*, *J Immunol.* 158:1796, 1997; Kawashima, 1. *et al.*, *Human Immunol.* 59:1, 1998); This procedure involves the stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a test peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks. T cells specific for the peptide become activated during this time and are detected using, eg., a Elispot assay involving peptide sensitized target cells.

2) Immunization of HLA transgenic mice (*see, e.g.*, Wentworth, P. A. *et al.*, *J Immunol.* 26:97, 1996; Wentworth, P. A, *et al.*, *Int Immunol.* 8:651, 1996; Alexander, J. *et al.*, *J Immunol.* 15 9:4753, 1997); In this method, peptides in incomplete Freund's adjuvant are administered subcutaneously to HLA transgenic mice. Several weeks following immunization, splenocytes are removed and cultured *in vitro* in the presence of test peptide for approximately one week. Peptide-specific T cells are detected using e.g., an Elispot assay involving peptide sensitized target cells and target cells expressing endogenously generated antigen.

3) Demonstration of recall T cell responses from patients who have been effectively vaccinated or who have a tumor; (see, e.g., Rehermann, B. et al., J *Exp. Med.*

181:1047, 1995; Doolan, D. L. et al., *Immunity* 7:97, 1997; Bertoni, R. et al., J Clin. Invest. 100:503, 1997; Threlkeld, S. C. et al., J *Immunol*. 159:1648, 1997; Diepolder, H. M. et al., J Virol. 71:6011, 1997; Tsang et al., J. *Natl*. Cancer Inst. 87:982990, 1995; Disis et al., J *Immunol*. 156:3151-3158, 1996). In applying this strategy, recall responses are detected by culturing PBL from patients with cancer who have generated an immune response "naturally", or from patients who were vaccinated with tumor antigen immunomodulatory preparations. PBL from subjects are cultured in vitro for 1-2 weeks in the presence of test peptide plus antigen presenting cells (APC) to allow activation of "memory" T cells, as compared to "naive" T cells. At the end of the culture period, T cell activity is detected using assays for T cell activity including an Elispot assay involving peptide-sensitized targets, T cell proliferation, or lymphokine release.

The methods of the invention can be applied to identifying peptides involved in the pathogenesis of or protection from any of a wide range of diseases, especially those in which relative susceptibility or resistance has been associated with expression of a particular class I MHC allele, provided that the amino acid sequence (or partial amino acid sequence) of a suspect polypeptide antigen is available. Candidate diseases include, without limitation, infectious diseases (e.g., diseases caused by *Chlamydia trachomatis, Helicobacter pylori, Neisseria meningitidis, Mycobacterium leprae, M. tuberculosis*, Measles virus, hepatitis C virus, human immunodeficiency virus, and *Plasmodium falciparium*), cancer (e.g. melanoma, ovarian cancer, breast cancer, colon cancer and B cell lymphomas)

1. Methods of Identifying $CD8^+$ T Cell Activating Peptide Epitopes Derived From Polypeptide Antigens

The methods of the invention have two distinct phases. The first is identification of epitopes that were specifically derived from a tumor associated antigen (TAA) and the second is "Epitope Verification" (EV). The purpose of the first stage of the process is to identify the individual peptide fragments generated by the proteasome complex of the source cell from the candidate polypeptide TAA. Any peptides of the appropriate length (about 8 to 15 amino acid residues), and having specific binding affinity for a particular class I MHC molecule expressed by the target cell or tissue, will bind to that class I MHC molecule in the endoplasmic reticulum. At least some of these peptide-class I MHC molecular complexes then migrate to the cell membrane of the cell. The complexes (both cell-membrane associated and intracellular) are isolated from the tissue/cell source and the peptides eluted from the complexes. The eluted peptides are then separated, their amino acid sequences determined, and the sequences compared to that of the candidate polypeptide.

The described method can generally be applied to the analysis of peptides produced by any tissue/cell source expressing defined class I MHC molecules. As such, the method can be useful for basic research studies, e.g., studies aimed at identifying amino acid residues in a polypeptide that determine sites of "cutting" by the proteolytic antigen processing enzymes of any tissue/cell source. Alternatively, where the polypeptide is suspected of being an antigen that activates $CD8^+$ T cells which cause

or promote a particular disease or mediate protection from a disease, the described method can be used to identify disease-related or protective peptide epitopes derived from the polypeptide. This information would be useful for basic research into the etiology of the disease, or as a basis for development of diagnostics, therapeutics, or immunomodulatory preparations for the disease.

A peptide whose amino acid sequence matches that of a region of the candidate polypeptide is likely to be one that activates $CD8^+$ T cells involved in the pathogenesis of or immunity to the relevant disease. Such a peptide can be subjected to the EV procedure in which its ability to activate $CD8^+$ T cells is assayed. Those peptides that activate $CD8^+$ T cells are identified as peptides that can mediate protection from disease or its pathogenic symptoms.

Once such a peptide is identified, it can be synthesized in large amounts, by chemical or recombinant techniques, and used in diagnostic assays similar to the EV procedures listed below. Relevant peptides could be used singly or in combination. Alternatively, expression vectors encoding such a peptide or a combination of such peptides can be used to transfect or transduce appropriate APC (see below), and these can be used in similar diagnostic assays.

Furthermore, multimers (e.g., dimers, trimers, tetramers, pentamers, or hexamers) of a class I MHC molecule associated with a peptide defined by the method of the invention and conjugated to a detectable label (e.g., a fluorescent moiety, a radionuclide, or an enzyme that catalyzes a reaction resulting in a product that absorbs or emits light of a defined wavelength) can be used to

quantify T cells from a subject (e.g., a human patient) bearing cell surface receptors that are specific for such complexes. Relatively high numbers of such T cells are likely to be diagnostic of a relevant disease or an indication that the T cells are involved in immunity to the disease. In addition, continuous monitoring of a patient's relative numbers of multimer-binding T cells can be useful in tracking the course of a disease or the efficacy of therapy. Such assays have been developed using tetramers of class I MHC molecules associated with an HIV-1-derived or an influenza virus-derived peptide [Altman et al. (1996), Science 274:94-96; Ogg et al. (1998), Science 279:2103-2106]. Such complexes could be produced by chemical cross-linking of purified class I MHC molecules assembled in the presence of a peptide of interest or by modification of already established recombinant techniques for the production of class I MHC molecules containing a single defined peptide.

1.1 Epitope Identification Method

In the Epitope Identification Method, a sufficient number of cells are harvested (~1 x 10^8 to 1 x 10^{10} depending on the expression of the target tumor antigen and the class I MHC molecules), and the class I MHC molecules of interest are isolated by any one of various methods known in the art, e.g., immunoprecipitation. They may be isolated by affinity chromatography by the method of Urban et al. (1994), PNAS 91, 1534.

Peptides bound non-covalently to the isolated class I MHC molecules are then eluted from the latter. A variety of methods known in the art can be used, for example, the method of Chicz et al. (1992), Nature 358:764-768; Chicz

and Urban, Immunology Today 15:155-160; Urban et al, Critical Reviews in Immunology 17:387-397 or the novel solid phase extraction protocol as in Example 1.

The eluted peptides are separated by one of a variety of possible chromatographic methods, e.g., reverse phase chromatography. All the resulting fractions that contain peptides are then individually analyzed by mass spectrometry, using settings that do not fragment the peptides. The peptides corresponding to all the "peaks" obtained on the mass spectra can then be subjected to individual amino acid sequence analysis. The sequences of the individual peptides can be obtained by means known to those in the art. They can, for example, be obtained by LC/MS/MS, using instrument settings resulting in the fragmentation of the peptides into small fragments that are analyzed by the mass spectrometer. The amino acid sequences of the peptides are then compared to that of the TAA. Those with a sequence identical to a region of the TAA are candidates for EV.

Alternatively, other approaches for deriving the amino acid sequences of individual peptides can be utilized. These methods invoke a second dimension of peptide separation prior to mass spectrometric analysis. This is often achieved by coupling a separation technique such as reversed phase HPLC to a mass analyzer (such as but not limited to a quadrupole ion trap, linear ion trap, triple quadrupole instrument, magnetic sector, Fourier transform ion cyclotron resonance, quadrupole time-of-flight, or a hybrid of these analyzers) though an electrospray interface. Fractions can be analyzed in a data dependent mode of operation. In this mode, mass peaks are

dynamically and automatically selected for isolation and fragmentation to yield amino acid sequences. No prior knowledge of novel signals is required for this mode of peptide sequencing. Alternatively, fractions can be analyzed in a target dependent mode of operation. In this mode, mass peaks are targeted for isolation and fragmentation to yield amino acid sequences. Prior knowledge of ion signals is required for this mode of peptide sequencing.

1.2 EV

The EV procedure involves testing of peptides identified by the described epitope identification method for their ability to (a) bind the class I MHC from which they were eluted and (b) activate various $CD8^+$ T cell populations. Peptides with amino acid sequences identical to those identified by the described method are synthesized. The synthetic peptides are then tested for their ability to bind the class I MHC from which they were eluted and activate $CD8^+$ T cells from (a) test subjects expressing the class I MHC molecule of interest. In diseases (e.g., cancer or infectious diseases without an autoimmune component), a pattern of responsiveness would indicate that the relevant peptide is an epitope that activates $CD8^+$ T cells that can mediate immunity to the disease or, at least, a decrease in the symptoms of the disease.

$CD8^+$ T cell responses can be measured by a variety of *in vitro* methods known in the art. For example, whole peripheral blood mononuclear cells (PBMC) can be cultured with and without a candidate synthetic peptide and their cytokine production responses measured by, e.g., Elispot

assay. Cytokines include, without limitation, interleukin-2 (IL-2), IFN-γ, IL-4, IL-5, TNF-α, interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), GM-CSF, RANTES, MIP-1α, MIP-1β and transforming growth factor β (TGFβ). Assays to measure them include, without limitation, ELISA, ELISPOT and bio-assays in which cells responsive to the relevant cytokine are tested for responsiveness (e.g., proliferation) in the presence of a test sample. Alternatively, cytokine production by $CD8^+$ lymphocytes can be directly visualized by intracellular immunofluorescence staining and flow cytometry.

Once peptide epitopes associated with a particular disease have been identified, the EV described above can be used as a diagnostic test for the disease. Thus, lymphocytes from a subject suspected of having or being susceptible to the disease can be tested by any of the described methods for a $CD8^+$ T lymphocyte response to one or more (e.g., 2, 3, 4, 5, 6, 10, 15, or 20) appropriate peptides. If a significant $CD8^+$ T lymphocyte is detected, it is likely that the subject has or will develop the disease. The disease can be, for example, cancer and the peptides can be derived from, for example, CEA. Appropriate peptides can be, for example, any of those listed below (e.g., those with SEQ ID NOS:1-8).

As an alternative to the above-described EV, peptides identified by the epitope identification method can be tested for their ability to bind to an appropriate class I MHC molecule by methods known in the art using, for example, isolated class I MHC molecules or cells transfected with nucleic acid molecules encoding class I MHC molecules. One such method is described in Example 2.

These binding assays can also be used to test the ability of the peptides to bind to alternative class I MHC molecules, i.e., class I MHC molecules other than those from which they were eluted using the described method of the invention. Once such alternative class I MHC molecules are shown to bind the peptide(s), the diagnostic methods of the invention (using such peptides) and therapeutic methods of the invention can be applied to subjects expressing such alternative class I MHC molecules.

1.3 Diseases and their associations with class I MHC genes

The methods of the invention can be applied to the analysis of peptides involved in diseases associated with expression of defined class I MHC molecules and in which pathology or protection is due to the action of activated $CD8^+$ T cells. Such diseases include, without limitation, certain infectious diseases, cancer, and autoimmune diseases.

Melanoma cell-specific $CD8^+$ T cells, which may be involved in protective immune responses to malignant melanoma, recognize tyrosinase epitopes presented by HLA-A2 class I molecules.

1.4 Species

The methods of the invention can be applied to diseases with the described characteristics in a wide range of mammalian species, e.g., humans, non-human primates, horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, hamsters, rats, and mice. They will preferably be applied to diseases of humans.

1.5 Class I MHC Molecules

Class I MHC molecules have been identified in multiple mammalian species. In some of these species, expression of a particular class I MHC molecule has been associated with a particular $CD8^+$ T cell-mediated diseases (see above). In humans, for example, the class I MHC molecules are designated HLA-A, HLA-B, and HLA-C and in mice, H-2D, H-2K and H-2L. In all species, there are multiple alleles of each gene.

1.6 Tissue/cell sources

Tissue/cell sources that can be used for the epitope identification methods of the invention can be any mammalian cell that expresses class I MHC molecules on its surface, e.g., those listed above for use in EV (B lymphocytes, macrophages, monocytes, dendritic cells, and, in humans, T cells). Tissue/cell sources can also be tumor cells, e.g., B cell lymphoma cells or adenocarcinoma cells. It is also not required that the cells constitutively express class I MHC molecules. Class I MHC can be induced (*in vitro* or *in vivo*) (e.g., by IFN-γ) in such cells. Alternatively, immortalized lines of such cells can be used.

1.7 Polypeptide Antigens

Polypeptide antigens that can be used with the epitope identification methods can be those with a known amino acid sequence or those in which at least part of the amino acid sequence is known. They can be polypeptides that themselves are known or suspected to be involved in the disease process or immunity to the disease (e.g., CEA in cancer) or they can be derived from microbial organisms known or suspected to be involved in the disease process (e.g., *M. leprae* in leprosy). Examples of other polypeptide antigens include the core and viral coat proteins of viruses such as hepatitis C virus, the heat shock proteins of mycobacteria, and tyrosinase in melanoma. Furthermore, the polypeptide antigen can be the full-length protein or it can be a fragment of the protein known or suspected to be involved in the disease process.

2. Peptides

Peptides of the invention include peptides that bind to class I MHC molecules and activate $CD8^+$ T cells involved in a disease process or protection from a disease. The class I MHC molecule can be a class I MHC molecule that is associated with susceptibility or resistance to a disease. Diseases can be any of the diseases cited herein and the species from which the class I MHC molecules and/or peptides are obtained can be any of those cited herein. The class I MHC molecules are preferably human class I HLA molecules, i.e., A, B or C molecules. The peptides can be, for example, peptides that bind to HLA-A2 molecules. The polypeptides from which the peptides of the invention are derived can be any of those cited herein. The peptides generally are 8 to 15 (e.g., 9 to 15) amino acids in length.

The peptides can be derived, for example, from CEA and can bind to HLA-A2 molecules. The peptides can be, for example, any one of the following peptides: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also included are peptides containing any of the above sequences, plus 1-15 residues on either or both ends.

In addition, peptides identified as being associated with any of the diseases listed herein (e.g., cancer such as colon cancer or any of the infectious diseases recited herein) can be used to activate lymphocytes (e.g., CD8+ lymphocytes) that cause, directly or indirectly, the death of pathogenic target cells such as cancer cells or

pathogen-infected cells. In addition, peptidomimetic forms of the peptides can be produced by methods known in the art. The peptides can be fragments of any the polypeptides disclosed herein, e.g., CEA or PSA. They can be, for example, those with SEQ ID NOS:1-8.

The CEA peptides of the invention (e.g., those with SEQ ID NOS:1-8), can be used for purposes other than therapy. They can be used, for example, in diagnostic assays and for methods of screening for reagents that bind to complexes of class I MHC molecules and CEA peptides (see below).

The peptides can be prepared using the described epitope identification methodologies. Smaller peptides (fewer than 50 amino acids long) can also be conveniently synthesized by standard chemical means. In addition, both polypeptides and peptides can be produced by standard *in vitro* recombinant nucleic acid techniques, and *in vivo* transgenesis using the nucleotide sequences encoding the appropriate polypeptides, peptides or APL. Methods well known to those skilled in the art can be used to construct expression vectors containing relevant coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook et al., Molecular Cloning: A Laboratory Manual [Cold Spring Harbor Laboratory, N.Y., 1989], and Ausubel et al., Current Protocols in Molecular Biology, [Green Publishing Associates and Wiley Interscience, N.Y., 1989].

The invention also features isolated nucleic acid molecules encoding the peptides of the invention. These nucleic acid molecules can be cDNA, genomic DNA, synthetic DNA, oligonucleotides, ribozymes or RNA, and can be

double-stranded or single-stranded (*i.e.*, either a sense or an antisense strand). Segments of these molecules are also considered within the scope of the invention, and can be produced, for example, by the polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription. Preferably, the nucleic acid molecules encode peptides that, regardless of length, are soluble under normal physiological conditions.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide (for example, the peptides with SEQ ID NOS:1-8). In addition, these nucleic acid molecules are not limited to coding sequences, e.g., they can include some or all of the non-coding sequences that lie upstream or downstream from a coding sequence.

The nucleic acid molecules of the invention can be synthesized (for example, by phosphoramidite-based synthesis) or obtained from a biological cell, such as the cell of a mammal. Thus, the nucleic acids can be those of a human, non-human primate (e.g., monkey) mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, dog, or cat.

In addition, the isolated nucleic acid molecules of the invention encompass segments that are not found as such in the natural state. Thus, the invention encompasses recombinant nucleic acid molecules, (for example, isolated nucleic acid molecules encoding any of the peptides described herein) incorporated into a vector (for example,

a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location).

Certain nucleic acid molecules of the invention are antisense molecules or are transcribed into antisense molecules. These can be used, for example, to down-regulate translation of CEA mRNA within a cell.

Techniques associated with detection or regulation of genes are well known to skilled artisans and such techniques can be used to diagnose and/or treat disorders associated with aberrant CEA expression, e.g., colon cancer. Hybridization can be used as a measure of homology between two nucleic acid sequences. Thus a nucleic acid encoding peptide of the invention (e.g., CEA peptides such as the peptide with SEQ ID NOs: 1-8), or a portion of such a nucleic acid, can be used as hybridization probe according to standard hybridization techniques. The hybridization of a CEA peptide probe to DNA or RNA from a test source (e.g., a mammalian cell) is an indication of the presence of CEA DNA or RNA, respectively, in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by one or more washes in 1 X SSC, 0.1% SDS at 50-60°C. Highly stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

The invention also encompasses: (a) vectors that contain any of the foregoing peptide coding sequences and/or their complements (that is, "antisense" sequence); (b) expression vectors that contain any of the foregoing peptide coding sequences operatively associated with any transcriptional/translational regulatory element necessary to direct expression of the coding sequences; (c) expression vectors containing, in addition to sequences encoding a peptide of the invention, nucleic acid sequences that are unrelated to nucleic acid sequences encoding the peptide of the invention, such as nucleic acid sequences encoding a reporter, marker, or a signal peptide (e.g., a heterologous signal peptide); and (d) genetically engineered host cells that contain any of the foregoing expression vectors and thereby express the nucleic acid molecules of the invention.

Where the nucleic acids form part of a hybrid gene encoding additional polypeptide sequences, for example, sequences that function as a marker or reporter, markers or reporter genes include β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, $G418^r$), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β-galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, additional sequences that can serve the function of a marker or reporter. Generally, the hybrid polypeptide will include a first portion and a

second portion, the first portion being a peptide of the invention (e.g., a peptide with any of SEQ ID NOS:1-8) and the second portion being, for example, a reporter described above or an immunoglobulin constant region.

A variety of host-expression vector systems can be used to express the peptides and polypeptides. Such host-expression systems represent vehicles by which the polypeptides of interest can be produced and subsequently purified, but also represent cells that can, when transformed or transfected with the appropriate nucleotide coding sequences, produce the relevant peptide or polypeptide *in situ*. These include, but are not limited to, microorganisms such as bacteria, e.g., *E. coli* or *B. subtilis*, transformed with recombinant bacteriophage nucleic acid, plasmid or cosmid nucleic acid expression vectors containing polypeptide coding sequences; yeast, e.g., *Saccharomyces* or *Pichia*, transformed with recombinant yeast expression vectors containing the appropriate coding sequences; insect cell systems infected with recombinant virus expression vectors, e.g., baculovirus; plant cell systems infected with recombinant virus expression vectors, e.g., cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV), or transformed with recombinant plasmid expression vectors, e.g., Ti plasmids, containing the appropriate coding sequences; or mammalian cell systems, e.g., COS, CHO, BHK, 293 or 3T3, harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells, e.g., metallothionein promoter, or from mammalian viruses, e.g., the adenovirus late promoter or the vaccinia virus 7.5K promoter.

The following examples are meant to illustrate, not limit, the invention.

Example 1. Identification of Class I MHC Binding CEA Peptide Epitopes

Materials and Methods

Carcinoma cell lines expressing CEA and HLA-A2. The carcinoma cell lines, KATO III, LS180, LS174T, and SW480, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained under the culture conditions recommended by the supplier. The cell lines were propagated in 2 L roller bottles to a density of ~10^6 cells/mL in RPMI 1640 medium supplemented with glutamine, penicillin/streptomycin and 10% fetal calf serum (FCS). All cell lines were tested for CEA surface expression using the murine mAb COL-1 (specific for human CEA) by FACS and for relative quantification by Western blot analysis. CEA expression was verified on all cell lines. Genotype analysis of each cell line confirmed HLA-A*0201 expression. Surface expression of HLA-A2 was verified using the murine mAb BB7.2.

HLA class I purification. Cells were harvested and pelleted by centrifugation. The cell pellets were weighed to determine the cellular mass and then frozen at -80°C prior to lysis. Each cell pellet was resuspended in lysis buffer (2 ml per gram of pellet) containing 1% CHAPS, 500 mM NaCl, 20 mM Tris-OH pH 8.0, in Milli-Q™ reverse osmosis quality (about 18.2 μΩ) water containing freshly added protease inhibitors (100 μM iodoacetamide, 8 μg/ml aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 5 mM EDTA, 0.04 % sodium azide, 1 mM PMSF), and the cells were

lysed by gentle agitation for 1 hr at 4°C on a rotor table. The resulting cell lysate was sedimented in an ultra-centrifuge for 1 hr at 230,000 x g at 4°C (37,500 RPM on a SW41 TI rotor). The insoluble material was removed by sedimentation at 175,000 x g for 2 hours, and the soluble supernatant fraction used for subsequent HLA purification. Multi-modal protein purification using HPLC columns was achieved by coupling the chromatographic sorbents in series with automated switching valves that direct the class I HLA-peptide complex containing effluent to subsequent columns in the sequences. The first three coupled columns were connected directly in series and acted together as a single pre-clearing column using high strength large throughpore perfusion sorbents (6000-8000 Å throughpores and 500-1000 Å diffusive pores, 50 µm) coated and crosslinked with a hydrophilic stationary phase and covalently conjugated with Protein A as the sorbent. These columns were designed to remove those proteins that non-specifically bind to the sorbents. Column 1 contained unmodified Protein A sorbent, column 2 contained Protein A coated with normal mouse serum, and column 3 was Protein A coated with bovine serum. The pre-clearing columns were followed by an immunoaffinity column of Protein A coupled with mAb specific for a non-polymorphic determinant on HLA-A2 molecules (A-BB7.2). After passing the lysate through the immunoaffinity column, the column was extensively washed with 25 column volumes of 0.1 % CHAPS/500 mM NaCl/0.05 % sodium azide/20 mM Tris-OH pH 8.0, followed by 25 column volumes of 0.1 % DOC/20 mM Mops/280 mM NaCl/0.05 % sodium azide pH 8.0, and finally 50 column volumes of 0.1 % DOC/0.05 % sodium azide/10 mM Tris-

OH pH 8.0. The HLA-A2-peptide complexes were eluted from the immunoaffinity support using 3.5 column volumes of 50 mM carbonate/0.1% DOC/0.05% NaN_3 at pH 11.5.

Peptide Analysis

The HLA class I protein samples can be concentrated by various methods. In one such method, the HLA class I protein sample eluted from the immunoaffinity column was concentrated to 100 µl using an ultrafiltration device (Amicon Centricon 3) prior to peptide extraction. Naturally processed peptide mixtures were acid eluted from HLA class I molecules by adding 800 µl 10 % acetic acid and incubating for 15 minutes at 70°C, as previously described [Chicz et al. (1993), J. Exp. Med. 178:24-47]. The peptides were separated from the remaining HLA protein by ultrafiltration with an Amicon Centricon 3™ device. The "flow-through" fraction containing the acid-extracted peptides was concentrated on a Savant SpeedVac™ centrifugal vacuum concentrator to a volume of approximately 20-100 µl and stored at -80°C. The acid-extracted peptide mixtures were then separated by reverse phase chromatography as previously described [Chicz et al. (1993), supra] but with minor modifications. Briefly, peptide solutions were preconcentrated by trapping the peptides using a small bed (0.5 - 3.0 µL bed volume) of polymeric reversed phase support. This also facilitates removal of hydrophilic contaminants that may be in the sample by washing the trap with a suitable aqueous solution (e.g., the buffers used for the chromatographic separation of the isolated peptides). Subsequently, peptides were back-flushed from the trapping phase to a microbore C18 column (1.0 x 250 mm; Vydac, Hesperia, CA), and peptide fractionation was

performed with a gradient of conventional solvents (containing water, acetonitrile and a suitable ion pair reagent) at a flow rate of 50 µl/minute. The column effluent was collected and stored at -20°C prior to analysis by mass spectrometry.

An alternative approach was to concentrate HLA class I molecule samples and extract the associated peptides using solid phase extraction (SPE). In this method, the HLA class I molecule samples were concentrated using a protein capture column that contained a suitable HPLC stationary phase. Appropriate HPLC stationary phases include a reversed phase (preferably a silica based C4, C8, C18 phase or a polymeric C4, C8 or C18-like reversed phase). However, a cation or anion exchange resin (including both strong, weak and mixed bed ion exchange phases) or other substrates that exhibit high affinity for protein complexes while enabling the elution of the HLA class I peptides, are equally suitable. Suitable dimensions of the protein capture column are 1-4.6 mm internal diameter and 2-5 cm long. Once the protein was bound to the capture column, the column was washed with suitable solvents (e.g., an aqueous buffer containing 5-25 mM Tris base at pH 7.5-8.5 followed by an aqueous solution of trifluroacetic acid (0.05-0.2% v/v)) to remove hydrophilic contaminants. The HLA class I molecule-peptide complexes were next disrupted by the action of a suitable solvent that also facilitated the elution of peptides from the protein capture column while leaving proteins adsorb on the stationary phase. An appropriate solvent for this purpose is a mixture of acetonitrile (5-25% v/v), and TFA (0.05-5% v/v) in water. The eluted peptides were collected

in a suitable container (e.g., an Eppendorf™ microfuge tube) and concentrated using a Savant Speedvac™ centrifugal vacuum concentrator. This step lowered the acetonitrile concentration and thereby permitted peptide fractionation by reverse phase chromatography (as described above). All peptide samples were stored at -20°C prior to analysis by mass spectrometry. Alternatively, the eluted peptides were mixed post-elution from the protein capture column with an aqueous solution of TFA (typically 0.05-0.2% TFA in water) in order to lower the acetonitrile concentration and thereby permit peptide adsorption onto a peptide capture column. The peptide capture column contained an HPLC phase (e.g., a reversed phase resin such as C18 or polymeric equivalent or an ion exchange resin or other suitable phase that exhibits high affinity towards peptides). When the peptide capture column contained an ion exchange resin (such as a strong cation exchanger) no solvent manipulation was required, as such phases efficiently adsorb peptides by charge, and or hydrophilic mechanisms that are not disrupted by relatively high levels of organic solvents. Suitable dimensions of the peptide capture column are 0.5-4.6 mm internal diameter and 1-5 cm long. Following the adsorption of peptides, the peptide capture column was washed with a suitable solvent (e.g., 0-5% acetonitrile, 0.05-0.2% TFA in water) prior to peptide fractionation by reversed phase chromatography (as described above). All peptide samples were stored at -20°C prior to analysis by mass spectrometry.

An automated microcapillary liquid chromatography-mass spectroscopy (LC-MS) approach with either targeted or data dependent collision-assisted dissociation (CAD) was used to

sequence low levels of naturally processed HLA associated peptides. Peptide fractions separated by reversed phase chromatography were diluted to aid handling and permit second dimension reversed phase separations. Typically, 1-5 µL aliquots of each peptide fraction were diluted to a final volume of 5-20 µL with a suitable solvent. A suitable solvent is one that is predominantly aqueous, that contains a low concentration 1-10 % by volume) of acetonitrile and an ion pair reagent such as TFA (at 0.1 - 1.0% by volume). Each diluted peptide solution was then concentrated by trapping peptides in a small bed (0.5 - 1.0 µL bed volume) of polymeric reversed phase support. This step also facilitated removal of hydrophilic contaminants by washing the trap with a suitable aqueous solution (e.g., mobile phase A as used in the chromatographic separation of isolated peptides). The peptides were then back flushed from the trapping phase onto the microcapillary (with an inner diameter of 75 µm and packed with 3-10 cm of 1-7 µm 100-300 Å C_{18} or non-porous material) and separation was developed using a non-linear gradient of conventional mobile phases for peptide separations (typically combinations of water and acetonitrile containing a suitable ion pair reagent). A mobile phase flow rate through the capillary column of 0.15-1.0 µL/min was achieved by splitting the flow from the pumps and using a backpressure regulator on the solvent waste line. Peptide detection was by µ-electrospray mass spectrometry. The voltage necessary to drive the electrospray was applied at the head of the microcapillary column (using a liquid junction interface) and peptides were electrosprayed into the mass analyzer directly as they eluted from the column.

CAD experiments were either predetermined to conduct specific target analyses or triggered in a data dependent mode, using ions that were more abundant than a user-set threshold. Dynamic exclusion was used in conjunction with data dependent analyses to ensure maximum peptide coverage (i.e., minor responses were analyzed by CAD following a user-determined number of CAD experiments of a single peptide response) by writing an exclusion list during assay progression so that a given ion will not be analyzed by multiple CAD experiments. The time that a given ion resides on the exclusion list was dependent upon the quality of the chromatographic separations. This time is determined experimentally. In this way, separated isobaric responses may be analyzed. Peptide sequencing sensitivities greater than 100 attomoles were achieved using this method. Alternatively, peptide fractions were reduced using microchemistries that convert oxidized methionine and cysteine containing peptides to their native forms. This chemistry requires the exchange of the acidic solution of reversed phase peptide fractions to be exchanged for a buffer of pH 8-9. This is conveniently achieved by concentrating each peptide fraction to dryness, and re-dissolving the peptide-containing residue in an ammonium bicarbonate buffer (100 mM in water at pH 8.2). The ammonium bicarbonate solution also contains a suitable reducing agent (such as 2-mercaptoethanol or dithiothreitol (DTT) or a combination of both reagents). The reducing agent is typically used at a concentration of 50 - 300 pmol/μL, and 5-20 μL of the reagent is sufficient for quantitative reduction of the peptide mixture. The reaction is typically performed at 37°C for 30 to 60 mins.

Subsequently the peptide fraction is reduced to dryness and re-dissolved in a solvent (as described above) that is suitable for peptide analysis by microcapillary LC/MS/MS. Reduced peptide fractions are analyzed as described for their non-reduced counterparts.

Results

Each preparation of tumor cells was grown to a density of ~10^6 cells/mL and harvested by sedimentation from 2 L roller bottles containing growth medium. Cell growth and viability varied among the cell lines. The three adherent cell lines were more difficult to consistently maintain in large scale culture and thus only SW480 was produced above the 20g threshold. The total amount of cells prepared were as follows: KATO III, 24.4g; SW480, 26.3g; LS174T, 17.6g; LS180, 12.3 g.

Protein purification was accomplished by immunoaffinity chromatography as described above. The HLA-A2 yields from the Protein-A-BB7.2 column for each preparation varied depending on the constituent expression levels of the target cell line and the amount of cell pellet processed. The HLA-A2 protein yields for the preparations listed above were as follows: KATO III, 40 ug; SW480, 19 ug; LS174T, 6 ug; and LS180, 2 ug.

Peptides were eluted from each HLA-A2 protein preparation and separated by RP-HPLC. The intact repertoire of peptides was separated into 100 fractions. RP-HPLC analysis was highly reproducible. Each fraction was analyzed by LC/MS/MS as described above in either a data dependent or targeted mass mode of operation.

Approximately 1050 analyses, including multiple analyses on the same sample, were conducted using LC/MS/MS

on the combined set of separated fractions. The overall analysis included both LC/MS and LC/MS/MS spectra collection. The initial preparation used for screening purposes was from the gastric carcinoma cell line, KATO III. Both triple play (MS, Zoom scan and MS/MS) and multiple MS/MS (MS, followed by three MS/MS scans) approaches were used to detect and identify novel HLA-A2 presented CEA epitopes from Kato III. Ions were automatically selected for MS/MS fragmentation during data acquisition as they exceeded an intensity threshold. Improved dynamic range was achieved by automatically placing precursor masses on an exclusion list for a period of time after their MS/MS spectrum was collected. Acquired MS/MS spectra were searched and analyzed using sequence analysis software and a CEA specific protein database. Peptides identified by this approach were subsequently targeted in the other carcinoma cell lines using LC/MS/MS.

Five CEA peptides (SEQ ID NOS:1-4 and 7)were originally identified and sequence verified (Figs. 1-4 and 6) from the KATO III cell line. The remaining two CEA peptides (SEQ ID NOS:6 and 8)were originally identified and sequence verified (Figs. 5 and 7) from LS180. The yields of individual CEA peptide epitopes varied during biochemical isolation and identification between the four carcinoma cell lines. Nevertheless, multiple epitopes were identified in each of the four carcinoma cell lines (Table 1).

Table 1: Summary of CEA epitope library identified from individual carcinoma cell lines (- denotes not detected, +\- denotes product ions consistent with sequence, but of

low abundance and + denotes epitope detection and approximate level).

Identified Peptide	KATO III	SW480	LS174T	LS180
SEQ ID NO:1	+	-	-	-
SEQ ID NO:2	++++			
SEQ ID NO:3	+++++	-	-	-
SEQ ID NO:4	+\-	-	-	+
SEQ ID NO:6	-	-	-	+\-
SEQ ID NO:7	+++	+++	+	+++
SEQ ID NO:8	-	+	+/-	++

Thus, the described method applied to the analysis of peptides produced by natural processing of CEA identified seven peptides that are associated with HLA-A2. This knowledge provides the basis for the development of therapeutic and/or prophylactic agents against CEA associated cancers, e.g., colon cancer. It is expected that analogous methodologies can be similarly successful in identifying other class I MHC-restricted tumor antigen peptides that activate $CD8^+$ T cells and are involved in the $CD8^+$ T lymphocyte-mediated pathogenesis of other diseases (see above).

Epitope Verification (EV):

Because tissue specific and developmental TAAs are expressed on normal tissue at least at some point in time or location within the body, it may be expected that T cells to them, particularly dominant epitopes, are eliminated during immunological surveillance and that tolerance is induced. However, $CD8^+$ T lymphocyte responses

to tumor epitopes in both normal donors and cancer patient has been detected, which may indicate that tolerance is incomplete *(see, e.g.,* Kawashima *et al., Hum.* Immunol. 59.1, 1998; Tsang, *J. Natl. Cancer Inst.* 87:82-90,1995; Rongcun *et al., J. Immunol. 163:1037, 1999). Thus,* immune tolerance does not completely eliminate or inactivate $CD8^+$ T lymphocyte precursors capable of recognizing high affinity HLA class I binding peptides.

Once HLA binding peptides are identified, they can be tested for the ability to elicit a T-cell response. Briefly, peptides comprising epitopes from a particular antigen are synthesized and tested for their ability to bind to the appropriate HLA proteins. These assays may involve evaluating the binding of a peptide of the invention to purified HLA class I molecules in relation to the binding of a radioiodinated reference peptide. Alternatively, cells expressing empty class I molecules *(i.e.* lacking peptide therein) may be evaluated for peptide binding by immunofluorescent staining and flow microfluorimetry. Other assays that may be used to evaluate peptide binding include peptide-dependent class I assembly assays and/or the inhibition of $CD8^+$ T lymphocyte recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for $CD8^+$ T lymphocytes derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or *in vivo* $CD8^+$ T lymphocyte responses that can give rise to populations capable of reacting with selected target cells associated with a disease.

Conventional assays utilized to detect T cell responses include proliferation assays, lymphokine secretion assays, direct cytotoxicity assays, and limiting dilution assays. For example, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce $CD8^+$ T lymphocyte responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells. Alternatively, mutant non-human mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides and that have been transfected with the appropriate human class I gene, may be used to test for the capacity of the peptide to induce *in vitro* primary $CD8^+$ T lymphocyte responses.

Peripheral blood mononuclear cells (PBMCs) may be used as the responder cell source of $CD8^+$ T lymphocyte precursors, The appropriate antigen-presenting cells are incubated with peptide, after which the peptide-loaded antigen-presenting cells are then incubated with the responder cell population under optimized culture conditions. Positive $CD8^+$ T lymphocyte activation can be determined by assaying the culture for the presence of $CD8^+$ T lymphocytes that kill radio-labeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed forms of the antigen from which the peptide sequence was derived.

More recently, a method has been devised which allows direct quantification of antigen-specific T cells by staining with Fluorescein-labelled HLA tetrameric complexes (Altman, J. D. *et al.*, *Proc. Natl. Acad Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996). Other

relatively recent technical developments include staining for intracellular lymphokines, and interferon- release assays or ELISPOT assays. Tetramer staining, intracellular lymphokine staining and ELISPOT assays all appear to be at least 10-fold more sensitive than more conventional assays (Lalvani, A. *et al, J Exp. Med* 186:859, 1997; Dunbar, P. R. *et al.*, Curr. Biol. 8:413, 1998; Murali-Krishna, K. *et al.*, *Immunity* 8: 177, 1998).

Alternatively, immunization of HLA transgenic mice can be used to determine immunogenicity of peptide epitopes. Several transgenic mouse models including mice with human HLA-A2 and B7 alleles have been characterized and others (*e.g.*, transgenic mice for HLA-A 1, -A3, -A11 and -A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed. Additional transgenic mouse models with other HLA alleles may be generated as necessary. Mice may be immunized with peptides emulsified in Incomplete Freund's Adjuvant and the resulting T cells tested for their capacity to recognize peptide-pulsed target cells and target cells transfected with appropriate genes. $CD8^+$ T lymphocyte responses may be analyzed using cytotoxicity assays described above.

To confirm that peptide epitopes identified are relevant to cancer (i.e., that they are recognized by $CD8^+$ T cells in the context of cancer), T cell recognition assays can be carried out using synthetic peptides having amino acid sequences based upon the sequence of the peptides identified by mass spectrometry to be derived from CEA (e.g., the peptides with SEQ ID NOS:1-4 and 6-8). Peptides can be synthesized using Fmoc chemistry and purified by RP-HPLC. The amino acid sequences and purity of greater than

90% for all the synthetic peptides can be confirmed by MALDI-MS and analytical HPLC. *In vitro* immunizations were performed with human lymphocytes from multiple leucopheresed healthy HLA-A2 donors. Peripheral blood mononuclear cells were obtained after a Ficoll-Hypaque gradient sedimentation. Effector cells were stimulated for 3 cycles at 7 days intervals. The peripheral lymphocytes were stimulated in the first cycle with dendritic cells (DC) and thereafter with peptide-pulsed adherent APC for the second and third cycle. DCs were pulsed 3 hours with 10ug/mL peptide in D-PBS, 1% BSA and 3ug/mL β-2m in a 24 well tissue plate and further cultured with peripheral blood lymphocytes (PBMC) ($2x10^6$/well). Cultures were restimulated with pulsed adherent cells on day 7 and day 14. IL-10 (10ng/mL) was added to the cultures 24 hours after each stimulation. IL-2 (10 IU/mL) was added to the cultures on days 9 and 16 and assays were performed day 21. HBV polymerase 455-463 (GLSRYVARL) peptide was used as the positive control.

T cell responses to CEA were analyzed by a IFN-gamma ELISPOT assay using a commercial IFN-gamma ELISPOT assay kit according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Briefly, effector cells were plated in 96 well plates in duplicate at $1x10^5$ cells/well. The cultures were stimulated with $1x10^5$ tumor target cells/well or T2 cells pulsed with 10ug/mL peptide. Each well of the 96-well hydrophobic PVDF membrane backed plate was previously absorbed with anti-IFN-γ monoclonal antibody (mAb) and blocked with 10% FCS for twenty minutes followed by an 24 hour incubation at 37°C in 5% CO_2. After which, each well

was washed four times and incubated overnight at 4°C with a biotinylated non-competing anti-IFN-γ mAb. Wells were washed three times, incubated for two hours at room temperature with streptavidin alkaline-phosphatase, washed again three times and developed with a thirty-minute incubation with BCIP/NBT and washed extensively with distilled water. IFN-γ^+ secreting cells (spots) were enumerated on an automated ELISPOT reader system with KS ELISPOT Software 4.2 (Zellnet Consulting, Inc., New York, NY).

Data obtained from the above EV analysis confirm that the peptides identified by the defined method are recognized specifically by $CD8^+$ T lymphocytes from five HLA-A2 expressing donors capable of targeting cancer cells expressing both HLA-A2 and CEA (Table 2).

Table 2: Summary of immunological verification for HLA-A2 presented CEA epitope library from five HLA-A2 donors (nt denotes not tested, (-) denotes not detected, and (+) denotes epitope detection and approximate level).

CEA epitope	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
SEQ ID NO:1	nt	nt	++	++++	++
SEQ ID NO:2	nt	nt	nt	++	nt
SEQ ID NO:3	nt	nt	++	++++	+
SEQ ID NO:4	-	++++	++	++	nt
SEQ ID NO:6	nt	nt	-	+	-
SEQ ID NO:7	+++	++++	++	++	nt
SEQ ID NO:8	++	+	-	++	nt

Example 2. Binding of peptides to isolated HLA-A2 molecules

The following example of peptide binding to HLA molecules demonstrates quantification of binding affinities of HLA class I peptides.

HLA class I binding assays using purified HLA-A2 molecules were performed in accordance with disclosed protocols (e.g., Sidney *et al.*, *Current Protocols* in Immunology 18.3.1 (1998); Sidney, *et al.*, J. Immunol. 154:247 (1995); Sette, *et al.*, *Mol. Immunol. 31:813* (1994)). Briefly, recombinant HLA-A2 molecules (5 to 500nM) were incubated with the ^{125}I radiolabeled HBV core 18-27 (FLPSDYFPSV) reference peptide and beta-2-microglobulin. The mixture was allowed to fold over a 48 hr incubation period. Stable HLA peptide complexes were separated from free peptide by size exclusion chromatography and the fraction of peptide bound was determined. Typically, in preliminary experiments, the HLA-A2 preparation was titered in the presence of fixed amounts of radiolabeled peptides to determine the concentration of HLA molecules necessary to bind 10-20% of the total radioactivity. All subsequent inhibition and direct binding assays were performed using these HLA concentrations.

Under these conditions the measured IC50 values are reasonable approximations of the true K_D values. Peptide inhibitors are typically tested at concentrations ranging from 120 ~g/ml to 1.2 ng/ml, and are tested in two to four completely independent experiments. To allow comparison of the data obtained in different experiments, a relative binding figure is calculated for each peptide by dividing the IC_{50} of a positive control for inhibition by the IC_{50} for each tested peptide (typically unlabeled versions of the radiolabeled probe peptide).

Peptides corresponding to the identified CEA epitopes were synthesized and tested in peptide binding experiments measuring the IC50 compared to a known HLA-A2 binding peptide. The results of the peptide binding assay are listed in Table 3. All seven identified CEA epitopes were capable of specific binding to HLA-A2. Five of the seven eptiopes bound with relatively high affinity with the remaining two epitopes representing weak binding affinity.

Table 3: IC50 results for CEA epitopes.

CEA epitope	IC50 (nM)
SEQ ID NO:1	22
SEQ ID NO:2	> 1000
SEQ ID NO:3	180
SEQ ID NO:4	81
SEQ ID NO:6	56
SEQ ID NO:7	27
SEQ ID NO:8	1122

Other Embodiments

The invention also features the following embodiments.

Methods of Use

Diagnostic Agents and for Evaluating Immune Responses

In one embodiment of the invention, HLA class I peptides as described herein are used as reagents to evaluate an immune response. The immune response to be evaluated is induced by using as an immunogen any agent that may result in the production of antigen-specific $CD8^+$ T lymphocytes that recognize and bind to the peptide epitope(s) to be employed as the reagent. The peptide reagent need not be used as the immunogen. Assay systems

that are used for such an analysis include relatively recent technical developments such as tetramers, staining for intracellular lymphokines and interferon release assays, or ELISPOT assays.

For example, peptides of the invention are used in tetramer staining assays to assess peripheral blood mononuclear cells for the presence of antigen-specific $CD8^+$ T lymphocytes following exposure to a tumor cell antigen or an immunogen. The HLA-tetrameric complex is used to directly visualize antigen-specific $CD8^+$ T lymphocytes (*see, e.g.*, Ogg *et al.*, *Science* 279:2103-2106, 1998; and Altman *et al.*, *Science* 174:94-96, 1996) and determine the frequency of the antigen-specific $CD8^+$ T lymphocyte population in a sample of peripheral blood mononuclear cells. A tetramer reagent using a peptide of the invention is generated as follows: A peptide that binds to an HLA molecule is refolded in the presence of the corresponding HLA heavy chain and β_2-microglobulin to generate a trimolecular complex. The complex is biotinylated at the carboxyl terminal end of the heavy chain at a site that was previously engineered into the protein. Tetramer formation is then induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to stain antigen-specific cells. The cells can then be identified, for example, by flow cytometry. Such an, analysis may be used for diagnostic or prognostic purposes. Cells identified by the procedure can also be used for therapeutic purposes.

Peptides of the invention are also used as reagents to evaluate immune recall responses (*see, e.g.*, Bertoni *et al.*, *J. Clin. Invest.* 100:503-513, 1997 and Penna *et al.*, *J*

Exp. Med. 174:1565-1570, 1991). For example, patient PBMC samples from individuals with cancer are analyzed for the presence of antigen-specific $CD8^+$ T lymphocytes using specific peptides. A blood sample containing mononuclear cells can be evaluated by cultivating the PBMCs and stimulating the cells with a peptide of the invention. After an appropriate cultivation period, the expanded cell population can be analyzed, for example, for $CD8^+$ T lymphocytes or for $CD4^+$ T lymphocyte activity.

The peptides are also used as reagents to evaluate the efficacy of an immunomodulatory preparation. PBMCs obtained from a patient vaccinated with an immunogen are analyzed using, for example, either of the methods described above. The patient is HLA typed, and peptide epitope reagents that recognize the allele-specific molecules present in that patient are selected for the analysis. The immunogenicity of the immunomodulatory preparation is indicated by the presence of epitope-specific $CD8^+$ T lymphocytes in the PBMC sample.

Immunomodulatory Compositions

Immunomodulatory preparations that contain an effective amount of one or more peptides a described herein are further embodiments of the invention. Once appropriately immunogenic epitopes have been defined, they can be sorted and delivered by various means, herein referred to as "immunomodulatory" compositions. Such immunomodulatory compositions can include, for example, peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres *(see, e.g.,* Eldridge, *et*

al., Mol. Immunol. 28:287-294, 1991: *Alonso et al., Vaccine* 12:299-306, 1994, Jones *et al., Vaccine* 13:675-681, 1995), peptide compositions contained in immune stimulating complexes (ISCOMS) (see, e.g., Takahashi *et al., Nature* 344:873-875, 1990; Hu *et al., Clin Exp Immunol.* 113:235-243, 1998), multiple antigen peptide systems (MAPs) *(see e.g.,* Tam, J. P., *Proc. Natl. Acad. Sci. USA.* 85:5409-5413, 1988; Tam, J.P., *J Immunol. Methods* 196:17-32, 1996), peptides formulated as multivalent peptides; peptides for use in ballistic delivery systems, typically crystallized peptides, viral delivery vectors (Perkus, M. E. *et al.,* In: *Concepts in vaccine development,* Kaufmann, S. H, E., ed., p, 379, 1996; Chakrabarti, S. *et al., Nature* 320:535, 1986; Hu, S. L. *et al., Nature* 320:537, 1986; Kieny, M.-P. *et al., AIDS Bio/Technology* 4:790, 1986; Top, F. H. *et al., J. Infect. Dis.* 124:148, 197 1; Chanda, P. K *et al., Virology* 175:535, 1990), particles of viral or synthetic origin *(e.g.,* Kofler, N. *et al., J Immunol. Methods.* 192:25, 1996; Eldridge, J. H. *et al., Sem. Hematol.* 30:16, 1993; Falo, L. D., Jr. *et al., Nature Med.* 7:649, 1995), adjuvants (Warren, H. S., Vogel, F. R., and Chedid, L. A. *Annul. Rev. Immunol.* 4:369,1986; Gupta, R. K. *et al., Vaccine* 11:293, 1993), liposomes (Reddy, R. *et al., J Immunol.* 148:1585, 1992; Rock, K. L., *Immunol. Today* 17:131, 1996), or, naked or particle absorbed cDNA (Ulmer, J. B. *et al., Science* 259:1745, 1993; Robinson, H. L., Hunt, L. A., and Webster, R. G., *Vaccine* 11:957, 1993; Shiver, J. W. *et al.,* In: *Concepts in vaccine development,* Kaufmann, S. H. E., ed., p. 423, 1996; Cease, K. B., and Berzofsky, J. A., *Annul, Rev. Immunol.* 12:923, 1994 and Eldridge, J. H. *et al., Sem. Hematol.* 30:16, 1993). Toxin-targeted delivery technologies,

also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) can also be used.

Immunomodulatory preparations of the invention include nucleic acid-mediated modalities. DNA or RNA encoding one or more of the peptides of the invention can also be administered to a patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,827,516; 5,880,103 and in more detail below. Examples of nucleic acid -based delivery technologies include "naked DNA", facilitated (bupivicaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or microparticle delivery (U.S. Patent No.5,783,567).

For therapeutic or prophylactic immunization purposes, the peptides of the invention can also be expressed by viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowl pox. As an example of this approach, vaccinia virus is used as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into a host bearing a tumor, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host $CD8^+$ T lymphocyte response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, *U.S.* Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover. *et al.*, *Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g. adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella*

typhi vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein.

Furthermore, immunomodulatory preparations in accordance with the invention encompass compositions comprising one or more of the claimed peptides. A peptide can be present in an immunomodulatory preparation individually. Alternatively, the peptide can exist as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides. Polymers have the advantage of increased immunological reaction and, where different peptide epitopes are used to make up the polymer, the additional ability to induce antibodies and/or $CD8^+$ T lymphocytes that react with different antigenic determinants of the pathogenic organism or tumor-related peptide targeted for an immune response. The composition can be a naturally occurring region of an antigen or can be prepared, *e.g.*, recombinantly or by chemical synthesis.

Carriers that can be used with immunomodulatory preparations of the invention are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly *L*-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like, The immunomodulatory preparations can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, preferably phosphate buffered saline. The immunomodulatory preparations also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are examples of materials well known in the art. Additionally, as disclosed herein, $CD8^+$ T

lymphocyte responses can be primed by conjugating peptides of the invention to lipids, such as tripalrnitoyl-S-glycerylcysteinlyseryl-serine (P_3CSS).

Upon immunization with an peptide composition in accordance with the invention, via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, the immune system of the host responds to the immunomodulatory preparation by producing large amounts of $CD8^+$ T lymphocytes specific for the desired antigen. Consequently, the host becomes at least partially immune to later infection, or at least partially resistant to developing an ongoing chronic infection, or derives at least some therapeutic benefit when the antigen was tumor-associated.

An immunomodulatory preparation of the invention can also include antigen-presenting cells (APC), such as dendritic cells (DC), as a vehicle to present peptides of the invention. Immunomodulatory preparation compositions can be created *in vitro*, following dendritic cell mobilization and harvesting, whereby loading of dendritic cells occurs *in vitro*. For example, dendritic cells are transfected, *e.g.*, with a minigene in accordance with the invention, or are pulsed with peptides. The dendritic cell can then be administered to a patient to elicit immune responses *in vivo*.

Immunomodulatory preparation compositions, either nucleic acid - or peptide-based, can also be administered *in vivo* in combination with dendritic cell mobilization whereby loading of dendritic cells occurs *in vivo*.

Antigenic peptides are used to elicit a $CD8^+$ T lymphocytes response *ex vivo*, as well. The resulting $CD8^+$ T

lymphocytes can be used to treat tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a therapeutic immunomodulatory peptide or nucleic acid in accordance with the invention. *Ex vivo* $CD8^+$ T lymphocyte response to a particular tumor-associated antigen are induced by incubating in tissue culture the patient's, or genetically compatible, $CD8^+$ T lymphocyte precursor cells together with a source of antigen-presenting cells, such as dendritic cells, and the appropriate immunogenic peptide. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded into effector cells, the cells are infused back into the patient, where they will destroy ($CD8^+$ T lymphocytes) their specific target cell (an infected cell or a tumor cell). Transfected dendritic cells may also be used as antigen presenting cells.

The immunomodulatory compositions of the invention can also be used in combination with other treatments used for cancer, including use in combination with immune adjuvants such as IL-2, IL-12, GM-CSF, and the like.

Preferably, the following principles are utilized when selecting an array of epitopes for inclusion in a polyepitope composition for use in an immunomodulatory preparation, or for selecting discrete epitopes to be included in an immunomodulatory preparation and/or to be encoded by nucleic acids such as a minigene. Exemplary epitopes that may be utilized in an immunomodulatory preparation to treat or prevent cancer include YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID

NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8). It is preferred that each of the following principles are balanced in order to make the selection. The multiple epitopes to be incorporated in a given immunomodulatory composition can be, but need not be, contiguous in sequence in the native antigen from which the epitopes are derived.

1.) Epitopes are selected which, upon administration, mimic immune responses that have been observed to be correlated with tumor clearance. For HLA Class I this includes 3-4 epitopes that come from at least one TAA. Epitopes from one TAA may be used in combination with epitopes from, one or more additional TAAs to produce an immunomodulatory preparation that targets tumors with varying expression patterns of frequently-expressed TAAs.

2.) When selecting epitopes from cancer-related antigens it is often useful to select analogs because the patient may have developed tolerance to the native epitope. When selecting epitopes for infectious disease-related antigens it is preferable to select either native or analog epitopes.

3.) Of particular relevance are epitopes referred to as "nested epitopes." Nested epitopes occur where at least two epitopes overlap in a given peptide sequence. A nested peptide sequence can comprise both HLA class I and HLA class II epitopes. When providing nested epitopes, a general objective is to provide the greatest number of epitopes per sequence. Thus, an aspect is to avoid providing a peptide that is any longer than the amino terminus

of the amino terminal epitope and the carboxyl terminus of the carboxyl terminal epitope in the peptide. When providing a multi-epitope sequence, such as a sequence comprising nested epitopes, it is generally important to screen the sequence in order to insure that it does not have pathological or other deleterious biological properties.

A number of different approaches are available which allow simultaneous delivery of multiple epitopes. Nucleic acids encoding the peptides of the invention are a particularly useful embodiment of the invention. Epitopes for inclusion in a minigene are preferably selected according to the guidelines set forth in the previous section. A preferred means of administering nucleic acids encoding the peptides of the invention uses minigene constructs encoding a peptide comprising one or multiple epitopes of the invention.

The immunogenicity of a multi-epitope minigene can be tested in transgenic mice to evaluate the magnitude of $CD8^+$ T lymphocyte induction responses against the epitopes tested. Further, the immunogenicity of nucleic acid - encoded epitopes in vivo can be correlated with the *in vitro* responses of specific $CD8^+$ T lymphocyte lines against target cells transfected with the nucleic acid plasmid. Thus, these experiments can show that the minigene serves to both: 1) generate a $CD8^+$ T lymphocyte response and 2) that the induced $CD8^+$ T lymphocytes recognized cells expressing the encoded epitopes. Persons skilled in the art can also refer to U.S. Patent Nos: 5,827,516; 5,880,103; 6,013,258; 6,183,746 and 5,783,567 and U.S.

Patent Application Nos: 09/321,346; 09/872,836; 60/262,219 (incorporated herein by reference) which describe the use of minigenes, nucleic acid encoded immunomodulatory preparations and delivery of such agents for therapeutic use.

For example, to create a nucleic acid sequence encoding the selected epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes may be reverse translated. A human codon usage table can be used to guide the codon choice for each amino acid. These epitope-encoding nucleic acid sequences may be directly adjoined, so that when translated, a continuous polypeptide sequence is created. To optimize expression and/or immunogenicity, additional elements can be incorporated into the minigene design. Examples of amino acid sequences that can be reverse translated and included in the minigene sequence include: HLA class I epitopes, HLA class II epitopes, a ubiquitination signal sequence, and/or an endoplasmic reticulum targeting signal. In addition, HLA presentation of $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte epitopes may be improved by including synthetic (eg. Poly-alanine) or naturally-occurring flanking sequences adjacent to the $CD8^+$ T lymphocyte or $CD4^+$ T lymphocyte epitopes; these larger peptides comprising the epitope(s) are within the scope of the invention.

The peptides of the present invention and pharmaceutical and immunomodulatory compositions of the invention are typically used therapeutically to treat cancer. Immunomodulatory compositions containing the peptides of the invention are typically administered to a cancer patient who has a malignancy associated with

expression of one or more tumor-associated antigens. Alternatively, immunomodulatory compositions can be administered to an individual susceptible to, or otherwise at risk for developing a particular type of cancer, *e.g.*, breast cancer.

In therapeutic applications, peptide and/or nucleic acid compositions are administered to a patient in an amount sufficient to elicit an effective $CD8^+$ T lymphocyte response to the tumor antigen and to cure or at least partially arrest or slow symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular composition administered, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

As noted above, peptides comprising $CD8^+$ T lymphocyte epitopes of the invention induce immune responses when presented by HLA molecules and contacted with a $CD8^+$ T lymphocyte specific for an epitope comprised by the peptide. The manner in which the peptide is contacted with the $CD8^+$ T lymphocyte is not critical to the invention. For instance, the peptide can be contacted with the $CD8^+$ T lymphocyte either in vivo or *in vitro*. If the contacting occurs *in vivo*, the peptide itself can be administered to the patient, or other vehicles, *e.g.*, nucleic acid vectors encoding one or more peptides, viral vectors encoding the peptide(s), liposomes and the like, can be used, as described herein.

When the peptide is contacted *in vitro*, the vaccinating agent can comprise a population of cells, *e.g.*, peptide-pulsed dendritic cells, or TAA-specific $CD8^+$ T lymphocytes, which have been induced by pulsing antigen-presenting cells *in vitro* with the peptide. Such a cell population is subsequently administered to a patient in a therapeutically effective dose.

For pharmaceutical compositions, the immunogenic peptides of the invention, or nucleic acid encoding them, are generally administered to an individual already diagnosed with cancer. The peptides or nucleic acid encoding them can be administered individually or as fusions of one or more peptide sequences. For therapeutic use, administration should generally begin at the first diagnosis of cancer. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. The embodiment of the immunomodulatory composition (i.e., including, but not limited to embodiments such as peptide cocktails, polyepitope polypeptides, minigenes, or TAA-specific $CD8^+$ T lymphocytes) delivered to the patient may vary according to the stage of the disease. For example, a immunomodulatory preparation comprising TAA-specific $CD8^+$ T lymphocytes may be more efficacious in killing tumor cells in patients with advanced disease than alternative embodiments.

The immunomodulatory compositions of the invention may also be used therapeutically in combination with treatments such as surgery. An example is a situation in which a patient has undergone surgery to remove a primary tumor and the immunomodulatory preparation is then used to slow or prevent recurrence and/or metastasis.

Where susceptible individuals, *e.g.*, individuals who may be diagnosed as being genetically pre-disposed to developing a particular type of tumor, are identified prior to diagnosis of cancer, the composition can be targeted to them, thus minimizing the need for administration to a larger population.

The dosage for an initial therapeutic immunization generally occurs in a unit dosage range where the value is about 1, 5, 50, or 500 µg. Dosage values for a human typically range from about 50 µg to about 500 µg per 70 kilogram patient. Boosting dosages of between about 50 µg to about 100 µg of nucleic acid pursuant to a boosting regimen over weeks to months may be administered depending upon the patient's response and condition as determined by measuring the specific activity of $CD8^+$ T lymphocyte obtained from the patient's blood.

Administration should continue until at least clinical symptoms or laboratory tests indicate that the tumor has been eliminated or that the tumor cell burden has been substantially reduced and for a period thereafter. The dosages, routes of administration, and dose schedules are adjusted in accordance with methodologies known in the art.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral, intrathecal, or local administration. Preferably, the pharmaceutical compositions are administered parentally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A

variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH-adjusting and buffering agents, tonicity adjusting agents, wetting agents, preservatives, and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monalaurate, triethanolamine oleate, *etc.*

The concentration of peptides of the invention in the pharmaceutical formulations can vary widely, *i. e.*, from less than about 0. 1 %, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, *in* accordance with the particular mode of administration selected.

A human unit dose form of the peptide composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, preferably an aqueous carrier, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans *(see, e.g.*, Remington's Pharmaceutical Sciences, 17" Edition, A. Gennaro, Editor, Mack Publishing Co., Easton, Pennsylvania, 1985).

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or to target selectively to infected cells, as well as to increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations, the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the peptide compositions. Liposomes for use in accordance with the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. Ile selection of lipids is generally guided by consideration *25 of, e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.* , Szoka, et *al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), and U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

For targeting cells of the immune system, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered

intravenously, locally, topically, *etc.* in a dose which varies according to, *inter alia*, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcurn, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1 %-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1 %-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In the cancer setting there are several findings that indicate that immune responses can impact neoplastic growth:

First, the demonstration in many different animal models, that anti-tumor T cells, restricted by MHC class I, can prevent or treat tumors.

Second, encouraging results have come from immunotherapy trials.

Third, observations made in the course of natural disease correlated the type and composition of T cell infiltrate within tumors with positive clinical outcomes (Coulie PG, et al. Antitumor immunity at work in a melanoma patient In Advances in Cancer Research, 213-242, 1999).

Finally, tumors commonly have the ability to mutate, thereby changing their immunological recognition. For example, the presence of monospecific $CD8^+$ T lymphocytes was also correlated with control of tumor growth, until antigen loss emerged (Riker A, et al., Immune selection after antigen-specific immunotherapy of melanoma Surgery, Aug: 126(2):112-20, 1999; Marchand M, et al., Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1 Int. J Cancer 80(2):219-3 0, Jan. 18, 1999). Similarly, loss of beta 2 microglobulin was detected in 5113 lines established from melanoma patients after receiving immunotherapy at the NCI (Restifo NP, el al., Loss of functional Beta-2-microglobulin in metastatic melanomas from five patients receiving immunotherapy Journal of the National Cancer Institute, Vol. 88 (2), 100-108, Jan. 1996). It has long been recognized that HLA class I is frequently altered in various tumor types. This has

led to a hypothesis that this phenomenon might reflect immune pressure exerted on the tumor by means of class I restricted $CD8^+$ T lymphocytes. The extent and degree of alteration in HLA class I expression appears to be reflective of past immune pressures, and may also have prognostic value (van Duinen SG, et al., Level of HLA antigens in locoregional metastases and clinical course of the disease in patients with melanoma Cancer Research 48, 1019-1025, Feb. 1988; Moller P, et al., Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma Cancer Research 51, 729-736, Jan. 1991). Taken together, these observations provide a rationale for immunotherapy of cancer and infectious disease, and suggest that effective strategies need to account for the complex series of pathological changes associated with disease.

Therapeutic Use in Cancer Patients

Evaluation of immunomodulatory compositions are performed to validate the efficacy of the $CD8^+$ T lymphocyte-$CD4^+$ T lymphocyte peptide compositions in cancer patients. The main objectives of the trials are to determine an effective dose and regimen for inducing $CD8^+$ T lymphocytes in cancer patients, to establish the safety of inducing a $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte response in these patients, and to see to what extent activation of $CD8^+$ T lymphocytes improves the clinical picture of cancer patients, as manifested by a reduction in tumor cell numbers. Such a study is designed, for example, as follows:

The studies are performed in multiple centers. The trial design is an open-label, uncontrolled, dose escalation protocol wherein the immunomodulatory

composition is administered as a single peptide dose followed six weeks later by a single booster shot of the same dose. The dosages are 500, 500 and 5,000 micrograms per injection. Drug-associated adverse effects (severity and reversibility) are recorded.

There are three patient groupings. The first group is injected with 50 micrograms of the immunomodulatory composition and the second and third groups with 500 and 5,000 micrograms of immunomodulatory peptide composition, respectively. The patients within each group range in age from 21-65, include both males and females (unless the tumor is sex-specific, e.g., breast or prostate cancer), and represent diverse ethnic backgrounds.

Induction of $CD8^+$ T lymphocyte Responses Using a Prime Boost Protocol

A prime boost protocol similar in its underlying principle to that used to evaluate the efficacy of a nucleic acid immunomodulatory preparation in transgenic mice, which was described in above, may also be used for the administration of the immunomodulatory preparation to humans. Such a therapeutic regimen may include an initial administration of, for example, naked or microencapsulated nucleic acid followed by a boost using recombinant virus encoding the immunomodulatory preparation, or recombinant protein/polypeptide or a peptide mixture administered in an adjuvant.

For example, the initial immunization may be performed using an expression vector, such as that described above, in the form of naked or microencapsulated nucleic acid administered IM (or SC or ID) in the amounts of 0.5-500 ug at multiple sites. The nucleic acid (0.1 to 500 ug) can also be administered using a gene gun. Following an incubation period of 3-4 weeks, a booster dose is then administered. The booster can be recombinant fowlpox virus

administered at a dose of $5x10^7$ to $5x10^9$ pfu. An alternative recombinant virus, such as an MVA, canarypox, adenovirus, or adeno-associated virus, can also be used for the booster, or the polyepitope protein, a mixture of the peptides or microencapsulated plasmid DNA encoding the epitopes can be administered. For evaluation of immunomodulatory efficacy, patient blood samples will be obtained before immunization as well as at intervals following administration of the initial immunomodulatory preparation and booster doses. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient sedimentation, aliquoted in freezing media and stored frozen. Samples are assayed for $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte activity.

Analysis of the results will indicate that a magnitude of response sufficient to achieve protective immunity against cancer is generated.

Adjunct Therapy to Surgery

The peptides of the invention can be used to activate $CD8^+$ memory T cells specific for CEA by *in vitro* methods known to those in the art. After a patient undergoes surgery to remove a tumor, such CEA-specific $CD8^+$ memory T cells may be administered to establish tumor-specific, long-lasting immunity against tumor rechallenge.

Reagents that Bind to Peptide-Class I MHC Complexes

Reagents that bind to peptide-class I MHC complexes can be made, for example, by screening a phage display library in which the phage particles contain nucleic acid sequences encoding antibody fragments such as Fab or single

chain Fv (scFv) fragments. Such libraries can be screened by testing for the presence of and isolating phage particles with the ability to bind to the peptides of the invention (e.g., YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), bound to a class I MHC molecule of interest. For example, phage display technology has been used to identify antibodies specific for defined HLA-A1 peptide complexes associated with melanoma [Chames et al. (2000), PNAS 97:7969-7974]. Alternatively, polyclonal antibodies or mAb can be screened for their ability to bind to peptide-class I MHC complexes of interest.

The above antibody-based reagents can be used, for example, in diagnosing cancer. For example, binding to a test cell of a Fab, scFv, or an antibody (e.g., a mAb) specific for a HLA-A2 molecule bound to a CEA peptide of the invention would indicate that the test cell is a cancer cell. Examples of detection agents used to detect binding of antibodies or antibody fragments include, without limitation, enzymes, radiolabels, luminescent compounds, and fluorescing compounds that elicit a detectable and measurable signal when the antibody complexes with the CEA naturally processed peptide. Examples of detectors include, without limitation, spectrophotometers, colorimeters, fluorometers, luminometers and biacore machines.

Therapeutic agents for treating cancer can be made, for example, by linking one of the above reagents with a therapeutic (e.g., cytotoxic) atom or molecule. An appropriate therapeutic agent, after administration (by any

of the methods disclosed herein) to a subject with the relevant cancer, binds to the cancer cells. The therapeutic atom or molecule can then kill the cancer cell. Alternatively, the therapeutic agent is internalized by the cancer cell and then the therapeutic atom or molecule kills the cancer cell. The linkage between the reagent and therapeutic atom or molecule can be a covalent one or a relatively weak non-covalent one such that the complex dissociates after binding to the cancer cell surface. Examples of therapeutic atoms and molecules include chemotherapeutic compounds, radioisotopes, and toxins, e.g., ricin or diptheria toxin, or toxic fragments of such toxins.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

Accordingly, the invention is limited only by the following claims.

1. A method of identifying a class I MHC-binding fragment of a polypeptide, the method comprising:

(a) isolating from the tissue/cell line a class I MHC molecule bound to a peptide, wherein the peptide is a class I MHC-binding fragment of the polypeptide;

(b) eluting the peptide from the class I MHC molecule; and

(c) identifying the peptide as a fragment of the polypeptide

2. The method of claim 1, wherein the polypeptide has the sequence of a tumor antigen.

3. The method of claim 1, wherein the tissue is a tumor.

4. The method of claim 1, wherein the cell line is a tumor cell line.

5. The method of claim 1, wherein the mammal is a human.

6. The method of claim 1, wherein the cell line is any mammalian cell that expresses class I MHC molecules on its surface.

7. The method of claim 1, wherein the class I MHC molecule is selected from the group consisting of a HLA-A molecule, a HLA-B molecule, and a HLA-C molecule.

8. The method of claim 1, wherein the class I MHC molecule is encoded by a gene selected from the group consisting of HLA-A1, HLA-A2, HLA-A3, HLA-A11, HLA-A24, HLA-B7, HLA-B8, HLA-B35, HLA-B44, HLA-B62 and HLA-B63.

9. The method of claim 1, wherein the class I MHC molecule is encoded by a gene selected from the group

consisting of A*0101, A*0201, A*0301, A*1101, A*2402, B*0702, B*0801, B*1502, B*3501, B*4401, B*5301 and B*5401.

10. A peptide fewer than 100 amino acids in length, the peptide comprising at least one isolated, naturally processed epitope consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8), wherein the peptide constitutes less than 70% of a naturally occurring CEA protein.

11. The peptide of claim 10, comprising at least two isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

12. The peptide of claim 10, comprising at least three isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

13. The peptide of claim 12, where the three isolated, naturally processed epitopes comprise the sequences ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI

(SEQ ID NO:8).

14. A peptide fewer than 100 amino acids in length, the peptide consisting of at least one isolated, naturally processed epitope consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8), wherein the peptide constitutes less than 70% of a naturally occurring CEA protein.

15. The peptide of claim 14, consisting of at least two isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

16. The peptide of claim 14, consisting of at least three isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

17. The peptide of claim 16, where the three isolated, naturally processed epitopes consist of the sequences ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI

(SEQ ID NO:8).

18. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNNQSL (SEQ ID NO:1).

19. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNDQSL (SEQ ID NO:2).

20. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNGQSL (SEQ ID NO:3).

21. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLSGANLNL (SEQ ID NO:4).

22. An isolated peptide fewer than 22 amino acid residues in length, comprising a sequence ATVGIMIGVLVGVALI (SEQ ID NO:5).

23. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence ATVGIMIGV (SEQ ID NO:6).

24. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence IMIGVLVGV (SEQ ID NO:7).

25. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence GVLVGVALI (SEQ ID NO:8).

26. The peptide library of isolated peptide fewer than 100 amino acid in length comprising the sequences YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8).

27. An altered peptide ligand (APL), the amino acid sequence of which is identical, except for 1-6 amino acid substitutions, to a fragment of carcinoembryonic antigen (CEA), the fragment being fewer than 20 amino acids residues in length and comprising a sequence selected from the group consisting of YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends, wherein no more than 30% of the amino acid residues of the fragment are substituted with different amino acid residues in the APL, and wherein the APL binds to a class I MHC molecule.

28. A process for making an APL, the process comprising:

a. carrying out the method of claim 1, and
b. synthesizing an APL consisting of a sequence which is identical to that of the peptide, except having amino acid substitutions at 1, 2, 3, 4, 5, or 6 positions in the peptide.

29. The process of claim 21, wherein the polypeptide is CEA.

30. A method of activating T cell reactivity in a mammal, the method comprising:

a. providing (i) a peptide, the sequence of which consists of the sequence of a naturally processed fragment of CEA, wherein the peptide binds to a class I MHC molecule of the mammal and elicits a $CD8^+$ T cell response, or (ii) a nucleic acid encoding a polypeptide selected from the group consisting of (1) the peptide, (2) the peptide plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and

b. administering the peptide or nucleic acid to the mammal.

31. The method of claim 23, wherein the peptide is YLWWVNNQSL (SEQ ID NO:1).

32. The method of claim 23, wherein the peptide is YLWWVNDQSL (SEQ ID NO:2).

33. The method of claim 23, wherein the peptide is YLWWVNGQSL (SEQ ID NO:3).

34. The method of claim 23, wherein the peptide is YLSGANLNL (SEQ ID NO:4).

35. The method of claim 23, wherein the peptide is ATVGIMIGVLVGVALI (SEQ ID NO:5).

36. The method of claim 23, wherein the peptide is ATVGIMIGV (SEQ ID NO:6).

37. The method of claim 23, wherein the peptide is IMIGVLVGV (SEQ ID NO:7).

38. The method of claim 23, wherein the peptide is GVLVGVALI (SEQ ID NO:8).

39. A method of altering a T cell response in a mammal, the method comprising:

a. providing (i) an APL having a sequence identical, except for amino acid substitutions at 1-6 positions, to the sequence of a naturally-processed fragment of CEA, wherein the APL binds to a class I MHC molecule of the mammal, or (ii) a nucleic acid encoding a polypeptide selected from the group consisting of (1) the APL, (2) the APL plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and

b. administering the APL or nucleic acid to the mammal.

33. The method of claim 1, further comprising:

(a) providing $CD8^+$ lymphocytes from a mammal having a condition suspected of being associated with presentation of the peptide by the class I MHC molecule, wherein the tumor cells of the mammal bear the class I MHC molecule;

(b) providing a population of tumor cells that bear the class I MHC molecule with the peptide bound thereto;

(c) contacting the population of tumor cells of (b) with the $CD8^+$ lymphocytes of (a); and

(d) determining whether the $CD8^+$ lymphocytes recognize the class I MHC-bound peptide, as an indication that presentation of the peptide to

$CD8^+$ T lymphocytes is associated with the condition.

34. The method of claim 33, wherein said presentation is associated with a pathological response of $CD8^+$ T lymphocytes.

35. The method of claim 33, wherein said presentation is associated with a protective response of $CD8^+$ T lymphocytes.

36. A method of diagnosis comprising:

(a) isolating an individual suspected of having or being susceptible to cancer;

(b) providing a $CD8^+$ lymphocyte from the individual;

(c) providing an APC which bears on its surface a class I MHC molecule of an allele identical to one expressed by said individual, wherein the class I MHC molecule is bound to a CEA peptide;

(d) contacting the APC with the $CD8^+$ lymphocyte; and

(e) determining whether the $CD8^+$ lymphocyte recognizes the class I MHC-bound peptide, as an indication that the individual has or is susceptible to cancer,

wherein the peptide comprises an amino acid sequence selected from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8).

37. A method of treating cancer, the method comprising:

(a) isolating a subject suspected of having or being susceptible to cancer; and

(b) administering the peptide of claim 10 to the subject.

38. A method of identifying a reagent for diagnosing cancer, the method comprising:

(a) providing a test reagent selected from the group consisting of a Fab fragment, a monoclonal antibody (mAb), and a single chain Fv (scFv) fragment;

(b) providing a complex comprising a class I MHC molecule bound to a peptide selected from the group consisting of YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8); and

(c) testing whether the test reagent binds to the complex.

39. The method of claim 38, wherein the class I MHC molecule is a HLA-A2 molecule encoded by a A*0201 gene.

40. A method of diagnosis, the method comprising:

(a) providing a test cell from a mammalian subject;

(b) providing a reagent that binds to a CEA peptide fragment bound to a class I MHC molecule;

(c) contacting the test cell with the reagent; and

(d) detecting binding of the reagent to the test cell as an indication that the test cell is a cancer cell.

41. A method of cancer treatment, the method comprising:

(a) isolating a subject suspected of having or being susceptible to cancer;

(b) providing a composition comprising a reagent selected from the group consisting of a Fab fragment, a mAb, and a scFv fragment, wherein the reagent recognizes a naturally processed CEA peptide bound to a MHC class I molecule, the reagent being linked to an agent selected from the group consisting of a chemotherapeutic compound, a radioactive isotope and a toxin; and

(c) administering the composition to the subject; wherein the cancer is characterized by expression of CEA and the administration results in an amelioration of one or more symptoms of the cancer.

42. A method of identifying a class I MHC-binding fragment of a tumor antigen, the method comprising:

(a) providing a mammalian tumour tissue or tumour cell line comprising a class I MHC molecule and the tumour antigen;

(b) isolating from the tissue/cell line the class I MHC molecule bound to a peptide, wherein the

peptide is a class I MHC binding fragment of the tumour antigen;

(c) eluting the peptide from the class I MHC molecule; and

(d) identifying the amino acid sequence of the peptide.

43. The method of claim 42, further comprising:

(e) providing $CD8^+$ lymphocytes from a mammal having a cancer suspected of being associated with presentation of the peptide by the class I MHC molecule, wherein the tissue/cell lines of the mammal bear the class I MHC molecule;

(f) providing a population of tumour cell line that bear the class I MHC molecule with the peptide bound thereto;

(g) contacting the population of tumour cell line of (f) with the $CD8^+$ lymphocytes of (e); and

(h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC bound peptide, as an indication that presentation of the peptide to $CD8^+$ lymphocytes is associated with the cancer.

44. An isolated nucleic acid comprising a nucleotide sequence encoding the peptide of claim 10 or a peptide of fewer than 20 amino acids in length and comprising the sequence of SEQ ID NOS:1-8.

45. A vector comprising the nucleic acid of claim 44.

46. The vector of claim 45, wherein the nucleotide sequence is operatively linked to a transcriptional regulatory element.

47. A cell comprising the vector of claim 45.

48. A cell comprising the vector of claim 46.

49. A method of enhancing an immune response to an antigen in animal comprising

(a) administering an effective amount of an inducing agent to the animal followed by

(b) administering an effective amount of the inducing agent and the antigen to the animal.

50. A method according to claim 49 where the inducing agent is a peptide or nucleic acid encoding a sequence from claim 10 and the antigen is CEA.

51. The peptide encoded by the nucleic acid of claim 44.

52. A polymeric delivery matrix selected from the group consisting of microspheres, hydrogels and polymeric networks and the nucleic acid of claim 44.

53. The polymeric delivery matrix of claim 52 where the matrix comprises a plurality of microspheres.

54. The microspheres of claim 53, wherein the polymeric matrix consists essentially of a polymer of poly-*co*- glycolic acid (PLGA).

55. A therapeutic composition comprising the nucleic acid of claim 44 and a pharmaceutically acceptable carrier.

56. The therapeutic composition of claim 56, further including an adjuvant.

57. A liposome comprising the nucleic acid of claim 44.

58. A method of eliciting an immune response in a mammal, which method comprises administering the nucleic acid of claim 44 to the mammal.

59. The method of claim 58, wherein the mammal is human.

60. The method of claim 59, wherein the pathogenic agent is CEA and the human suffers from, or is at risk of, cancer.

61. The method of claim 58, wherein the nucleic acid is administered directly to a mucosal tissue of the mammal.

62. The method of claim 58, wherein the nucleic acid is administered subcutaneously or intramuscularly.

63. A method of eliciting an immune response in a mammal, which method comprises administering the microspheres of claim 53 to the mammal

64. A method of treating a subject suspected of having

or being susceptible to cancer, the method comprising isolating the subject and administering the nucleic acid of claim 44 to the subject, wherein the administration results in an amelioration of one or more symptoms of cancer.

65. The method of claim 64, wherein administering the nucleic acid results in a decrease in a tumour size or activity.

Fig. 1

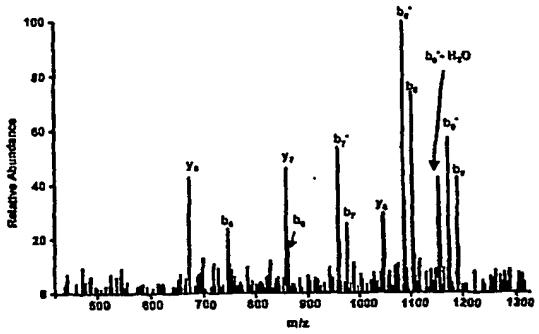


Fig. 2

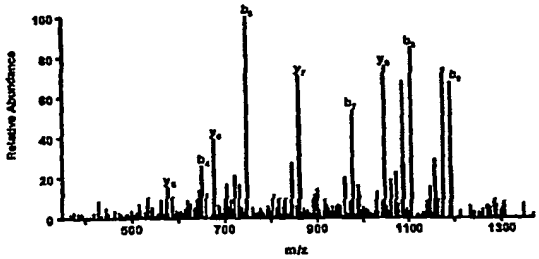


Fig. 3

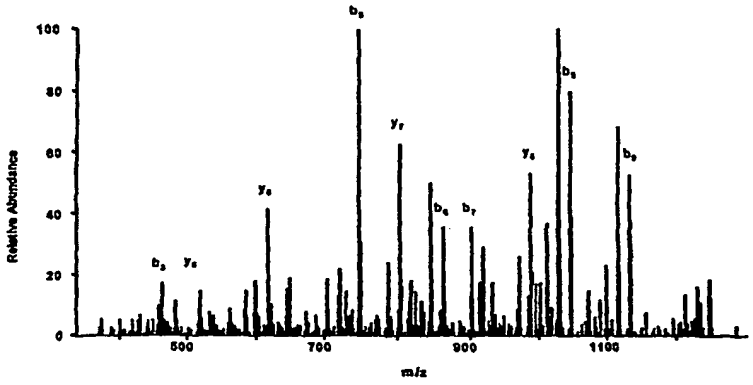
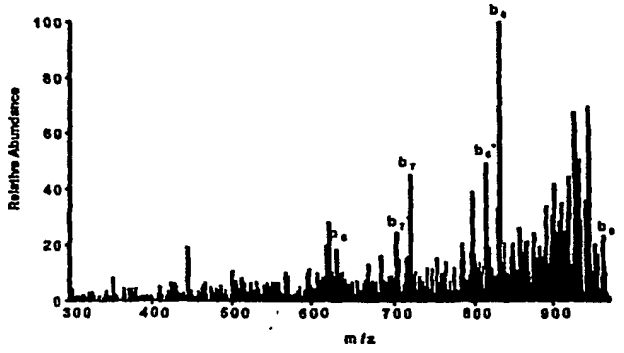


Fig.4



5

Fig 5

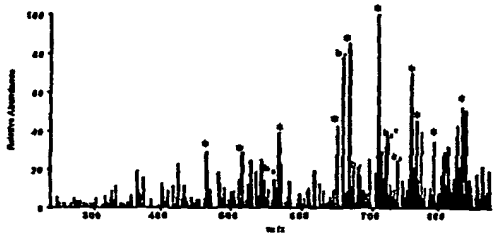
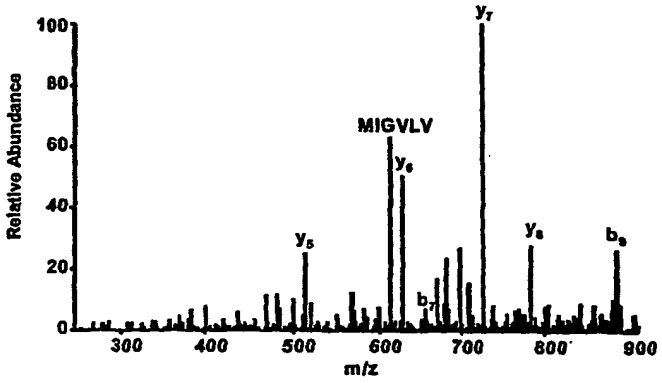


Fig 6


100
80
60
40
20
0
Relative Abundance
MIGVLV
y_7
y_6
y_5
y_4
b_8
b_7
300
400
500
600
700
800
900
m/z

5

Fig 7

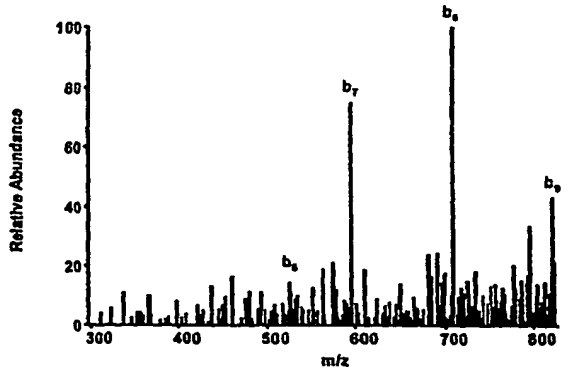


Fig 8.

MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE VLLLVHNLPQ
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TLHVIKSDLV NEEATGQFRV YPELPKPSIS SNNSKPVEDK DAVAFTCEPE TQDATYLWWV
NNQSLPVSPR LQLSNGNRTL TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP
TISPLNTSYR SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ NTTYLWWVNN
QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS VDHSDPVILN VLYGPDDPTI
SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL IDGNIQQHTQ ELFISNITEK NSGLYTCQAN
NSASGHSRTT VKTITVSAEL PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS
LPVSPRLQLS NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN GTYACFVSNL
ATGRNNSIVK SITVSASGTS PGLSAGATVG IMIGVLVGVA LI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/08427

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/04; C07K 7/00
US CL : 530/300, 327,328; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/300, 327,328; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG-Medline, Embase, Cancerlit, Scisearch, Biosis; BRS/EAST-USPatfull, EPO, JPO, Derwint, PGPubs

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 6,319,496 B1 (PANICALI et al.) 20 November 2001 (20.11.01), see entire document, particularly, Table 7 and claims 1-16.	10,14,21,30,34,44-51,55-56,58-62,64-65 ——— 11-13, 15-20, 22-29, 31-33, 35-41, 52-54, 57, 63
X — Y	US 6,001,349 (PANICALI et al.)14 December 1999 (14.12.99), see entire document, especially Table 7 and claims 1-12.	10,14,21,30,34,44-51,55-56,58-62,64-65 ——— 11-13, 15-20, 22-29, 31-33, 35-41, 52-54, 57, 63
X	HUNT. D. F. et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science. March 1992, Vol. 255, 1261-1263, see entire document.	1-9, 42-43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"E" earlier application or patent published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	RAS. E. et al. Identification of potential HLA-A 0201 Resricted CTL epitopes Derived from the epithelial cell adhesion molecule (Ep-CAM) and the carcinoembryonic antigen (CEA). Human Immunol. 1997, Vol. 53, pages 81-89, see entire document.	10,14,21,30,34,51 ——— 11-13, 15-20, 22-29, 31-33, 35-41, 44-50, 52-64
X	HUANG. A. Y.C. et al. The immunodominant major histocompatibility complex class I restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. PNAS. 1996, Vol. 93, pages 9730-9735, see entire document.	1-9, 42-43

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Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
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(54) Title: PEPTIDE EPITOPES RECOGNIZED BY ANTIGEN SPECIFIC CD8+ T LYMPHOCYTES

(57) Abstract: The invention provides methods for identifying and validating epitopes that are bound to class I MHC molecules and activate CD8+ T cells involved in the pathogenesis of or protection from diseases, e.g., cancers. The invention includes peptide epitopes derived from the CEA polypeptide by such methods, and methods of therapeutic use of these epitopes against diseases such as cancers.

WO 2003/082317 A1

PEPTIDE EPITOPES RECOGNIZED BY ANTIGEN SPECIFIC CD8$^+$ T LYMPHOCYTES

Field of the Invention

The invention relates to the identification of naturally processed and presented HLA class I restricted peptides and their use as therapeutics and prophylactics.

Background of the Invention

After proteolytic processing of intact protein antigens by antigen presenting cells (APCs), class I Major Histocompatibility Complex (MHC) molecules on the APCs bind short antigenic peptides (epitopes) derived from the antigens, presenting the bound peptides to CD8$^+$ T lymphocytes [Germain and Margulies (1993), Ann. Rev. Immunol. 11:403-450]. Class I MHC genes and the molecules they encode are highly variable among individuals, and differences among the class I MHC molecules determine which peptides are selected for presentation as T cell epitopes. Identification of the naturally processed peptide fragments of a polypeptide of interest that are presented by class I MHC molecules of a subject can be useful for developing peptides that regulate immune response to the polypeptide in that subject.

A growing body of evidence suggests that CD8$^+$ T lymphocytes are important in the immune response to tumor cells. CD8$^+$ T lymphocytes recognize peptide epitopes in the context of HLA class I molecules that are expressed on the surface of almost all nucleated cells. Following intracellular processing of endogenously synthesized tumor

antigens, antigen-derived peptide epitopes bind to class I HLA molecules in the endoplasmic reticulum. The resulting complex is transported to the cell surface. $CD8^+$ T lymphocytes recognize the peptide-HLA class I complex, which results in the destruction of the cell bearing the HLA-peptide complex directly by the $CD8^+$ T lymphocytes and/or via the activation of non-destructive mechanisms, *eg.*, activation of lymphokines such as tumor necrosis factor -α (TNF -α) or interferon- γ (IFN γ) which enhance the immune response and facilitate the destruction of the tumor cell.

A fundamental challenge in the development of an efficacious tumor immunomodulatory preparation is immune suppression or tolerance that can occur. There is therefore a need to establish immunomodulatory embodiments that elicit immune responses of sufficient breadth and vigor to prevent progression and/or clear the tumor. Persons skilled in the art can also refer to U.S. Patent Application 60/232,185 (incorporated herein by reference) which describes identification and use of $CD4^+$ T lymphocytes CEA peptide epitopes.

The epitope approach employed in the present invention represents a solution to this challenge, in that it allows the incorporation of $CD8^+$ T lymphocytes epitopes, from discrete regions of a target tumor-associated antigen (TAA) in a single immunomodulatory composition. Such a composition can simultaneously target multiple dominant and subdominant epitopes and thereby be used to achieve effective immunization in a diverse population.

Carcinoembryonic antigen (CEA) was first described by Gold and Freedman (1965), J. Exp. Med. 121:439-462. CEA is

a highly glycosylated, 180,000-dalton protein that is expressed on most gastrointestinal carcinomas, including colon, rectal, pancreatic and gastric (Muraro *et al.*, *Cancer Res.* 45:5769-5780, 1985) as well as 50% of breast (Steward *et al.*, *Cancer (Phila)* 33:1246-1252, 1974) and 70% of non-small cell lung carcinomas (Vincent et *al.*, *J. Thorac. Cardiovasc. Surg.* 66:320-328, 1978). It is also found on some normal, endodermally derived tissues, though in much lower concentrations. The abnormally high expression on cancer cells makes CEA an important target for immunotherapy. CEA cell surface expression can be detected using the monoclonal antibody (mAb) Col-1, and CEA can be identified in total cellular protein by western blot analysis using the same mAb. An MHC class I (HLA-A2)-restricted $CD8^+$ T lymphocytes response against a processed CEA epitope has been described in patients with metastatic disease [Tsang et al. (1995), J. Natl. Cancer. Inst. 87:982-990]. This MHC class I-restricted epitope was identified by taking peripheral blood lymphocytes from patients after immunization with a recombinant vaccinia virus encoding CEA and screening the T cell response against synthetic 9- to 11-mer CEA peptides. $CD8^+$ T lymphocytes from three patients were shown to have specificity for a class I-restricted CEA epitope in an assay using autologous Epstein Barr Virus (EBV)-transformed B cells pulsed with a 9 amino acid synthetic peptide derived from CEA.

SUMMARY OF THE INVENTION

The invention features methods for identifying peptide epitopes that activate $CD8^+$ T lymphocyte responses involved

in the initiation, promotion, or exacerbation of certain diseases.

The invention is based on the discovery that one can identify HLA class I-restricted epitopes naturally produced in tumor cell lines. These peptide epitopes can be incorporated into a drug delivery system and used to elicit an epitope-specific $CD8^+$ T cell response in an appropriate mammalian subject. Where the epitope is derived from a tumor antigen, such a response promotes the migration of $CD8^+$ T lymphocytes to the vicinity of tumor cells expressing the tumor antigen and specific targeting of the tumor cells by the $CD8^+$ T lymphocytes.

More specifically, the invention features a method of identifying a class I MHC-binding fragment of a polypeptide. The method involves: (a) isolating from tumor cell lines a class I MHC molecule bound to a peptide, the peptide being a class I MHC-binding fragment of the polypeptide; (b) eluting the peptide from the class I MHC molecule; (c) identifying the peptide as a fragment of the polypeptide; (d) chemical verification of the peptide by HPLC elution time mapping and LC/MS/MS fragmentation comparison to a synthetic homologue; (e) measuring the binding affinity of the peptide to the specific class I MHC molecule; and (e) immunological validation by stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a synthetic peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks, followed by specific recognition of the target tumor cells by the peptide specific generated $CD8^+$ T cells. The polypeptide can have the sequence of a tumor antigen. The tumor cells can be any class I MHC-expressing mammalian

cell, e.g., gastric carcinoma, colorectal adenocarcinoma, breast carcinoma, lung carcinoma, myeloma, glioma, or lymphoma, and the mammal from which it is derived can be a human. The class I MHC molecule can be a HLA-A molecule, or a HLA-B molecule, or a HLA-C molecule. Such a HLA-A molecule can be encoded by a A*0101, A*0201, A*0301, A*1101, or A*2402 gene.

The invention provides an isolated peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNDQSL (SEQ ID NO:2), where the aspartic acid at position P7 is a post translationally deamidated asparagine residue at P7 of (SEQ ID NO:1). Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNGQSL (SEQ ID NO:3), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLSGANLNL (SEQ ID NO:4), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGVLVGVALI (SEQ ID NO:5), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer

than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGV (SEQ ID NO:6), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence IMIGVLVGV (SEQ ID NO:7), optionally with additional CEA sequence on one or both ends. Also embraced by the invention is a peptide fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also embraced by the invention are altered peptide ligands (APL), which are class I MHC-binding variant peptides in which 1 to 6 amino acid residues are different from the corresponding residues of the wild-type class I MHC-binding peptide, but which still bind to the same class I MHC molecules as the wild-type peptides, corresponding to SEQ ID NOS:1-8, as are methods of therapy and prophylaxis involving the use of APL. APL have the ability to elicit different patterns of cytokine production in T cells than do their parent wild-type peptides. Thus, for example, while a wild-type peptide presented by a MHC molecule may induce production of Th1 cytokines, an APL derived from it and presented by the same MHC molecule may elicit Th2 or other immunoregulatory cytokines. Alternatively, the wild-type peptide may stimulate the production of Th2 cytokines while a corresponding APL elicits production of Th1 cytokines.

Another aspect of the invention is a method of activating T cell reactivity in a mammal. The method

involves: (a) providing (i) at least one peptide, the sequence of which consists of the sequence of a naturally processed fragment of CEA, the peptide being capable of binding to a class I MHC molecule of the mammal and of eliciting a $CD8^+$ T cell response, or (ii) a nucleic acid encoding a polypeptide that is (1) the peptide, (2) the peptide plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and (b) administering the peptide or nucleic acid to the mammal. The peptide can include or be YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The above described method of identifying a class I MHC-binding fragment of a polypeptide can include the additional steps of: (e) providing $CD8^+$ lymphocytes from a mammal having a condition suspected of being associated with presentation of the peptide by the class I MHC molecule, the APCs of the mammal bearing the class I MHC molecule;(f) providing a population of APCs that bear the class I MHC molecule with the peptide bound thereto; (g) contacting the population of APCs of (f) with the $CD8^+$ lymphocytes of (e); and (h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC-bound peptide, as an indication that presentation of the peptide to $CD8^+$ T lymphocytes is associated with the condition. The presentation can be associated with a pathological response of $CD8^+$ T lymphocytes or a protective response of $CD8^+$ T lymphocytes.

Another aspect of the invention is a method of diagnosis that involves: (a) providing a $CD8^+$ lymphocyte from an individual suspected of having or being susceptible to cancer; (b) providing an APC which bears on its surface a class I MHC molecule of an allele identical to one expressed by the individual, the class I MHC molecule being bound to a CEA peptide; (c) contacting the APC with the $CD8^+$ lymphocyte; and (d) determining whether the $CD8^+$ lymphocyte recognizes the class I MHC-bound peptide, as an indication that the individual has or is susceptible to cancer. The peptide can include or be the amino acid sequence YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The invention also features a method of treating a subject suspected of having or being susceptible to cancer. The method involves administering a peptide or isolated nucleic acid to the subject. The peptide or peptide encoded by isolated nucleic acid can be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNDQSL (SEQ ID NO:2), where the aspartic acid at position P7 is a post translationally deamidated asparagine residue at P7 of (SEQ ID NO:1). Alternatively, the peptide could be fewer than

17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLWWVNGQSL (SEQ ID NO:3), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length that includes or is the sequence YLSGANLNL (SEQ ID NO:4), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGVLVGVALI (SEQ ID NO:5), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence ATVGIMIGV (SEQ ID NO:6), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence IMIGVLVGV (SEQ ID NO:7), optionally with additional CEA sequence on one or both ends. Alternatively, the peptide could be fewer than 17 (e.g., fewer than 14, 12, 10, or 9) amino acid residues in length that includes or is the sequence GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends.

The invention also provides a method of identifying a reagent for diagnosing cancer. The method involves: (a) providing a test reagent that can be a Fab fragment, a monoclonal antibody (mAb), or a single chain Fv (scFv) fragment; (b) providing a complex that contains a class I MHC molecule bound to a peptide that includes or is

YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends; and (c) testing whether the test reagent binds to the complex. The class I MHC molecule can a HLA-A2 molecule encoded by a A*0201 gene.

Also embraced by the invention is a method of diagnosis. The method involves:(a) providing a test cell from a mammalian subject; (b) providing a reagent that binds to a CEA peptide fragment bound to a class I MHC molecule; (c) contacting the test cell with the reagent; and (d) detecting binding of the reagent to the test cell as an indication that the test cell is a cancer cell.

The invention also features a method of cancer therapy that involves: (a) providing a composition that includes a reagent that can be a Fab fragment, a mAb, or a scFv fragment, the reagent being able to recognize a naturally processed CEA peptide bound to a MHC class I molecule and being linked to an agent that can be a chemotherapeutic compound, a radioactive isotope or a toxin; and (b) administering the composition to a subject suspected of having or being susceptible to a cancer characterized by expression of CEA.

Another aspect of the invention is a method of identifying a class I MHC-binding fragment of a tumour antigen. The method involves: (a) providing a mammalian cell that contains a class I MHC molecule and the tumour antigen; (b) isolating from the cell the class I MHC molecule bound to a peptide, the peptide being a class I MHC binding fragment of the tumour antigen;(c) eluting the

peptide from the class I MHC molecule; and (d) identifying the amino acid sequence of the peptide. The method can involve the additional steps of: (e) providing $CD8^+$ lymphocytes from a mammal having a cancer suspected of being associated with presentation of the peptide by the class I MHC molecule, the cells of the mammal bearing the class I MHC molecule; (f) providing a population of cells that bear the class I MHC molecule with the peptide bound thereto; (g) contacting the population of cells of (f) with the $CD8^+$ lymphocytes of (e); and (h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC bound peptide, as an indication that presentation of the peptide to $CD8^+$ lymphocytes is associated with the cancer.

The invention also provides an isolated nucleic acid that contains a nucleotide sequence that encodes a peptide fewer than 17 (e.g., fewer than 13, 12, 10, or 9) amino acid residues in length and including the sequence YLWWVNNQSL (SEQ ID NO:1), optionally with additional CEA sequence on one or both ends. The peptide encoded by the nucleic acid thus can include or be the amino acid sequence YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also featured by the invention is a vector containing nucleic acid that encodes one or more of the peptides that are defined above. In the vector the nucleotide sequence can be operatively linked to a transcriptional regulatory element. Also included in the invention is a cell (e.g., a mammalian, an insect, a bacterial, a yeast, or a fungal cell) containing any of the vectors of the invention.

An "isolated" peptide of the invention is a peptide which either has no naturally-occurring counterpart (e.g., an APL), or has been separated or purified from components which naturally accompany it, e.g., in tissues such as pancreas, liver, spleen, ovary, testis, muscle, joint tissue, neural tissue, gastrointestinal tissue, or body fluids such as blood, serum, or urine. The peptide is considered "isolated" when it is at least 70%, by dry weight, free from the proteins and peptides with which it is naturally associated. Preferably, a preparation of a peptide of the invention is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, the peptide of the invention. Since a peptide that is chemically synthesized is, by its nature, separated from the components that naturally accompany it, the synthetic peptide is, by definition, "isolated."

An isolated peptide of the invention can be obtained, for example, by extraction from a natural source (e.g., from human tissues or bodily fluids); by expression of a recombinant nucleic acid encoding the peptide; or by chemical synthesis. A peptide that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will be separated from components that naturally accompany it. The extent of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, mass analysis, or HPLC analysis.

An "isolated nucleic acid " means nucleic acid free of the genes that flank the gene of interest (e.g., the gene encoding CEA) in the genome of the organism in which the

gene of interest naturally occurs. The term therefore includes a recombinant nucleic acid incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote. It also includes a separate molecule such as: a cDNA where the corresponding genomic DNA has introns and therefore a different sequence; a genomic fragment; a fragment produced by polymerase chain reaction (PCR); a restriction fragment; a nucleic acid encoding a non-naturally occurring protein, fusion protein, or fragment of a given protein; or a nucleic acid which is a degenerate variant of a naturally occurring nucleic acid. In addition, it includes a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Also included is a recombinant nucleic acid that includes a nucleic acid sequence that encodes any of the peptides with SEQ ID NOS:1-8.

As used herein, "protection from a mammalian disease" means prevention of onset of a mammalian disease or lessening the severity of a disease existing in a mammal. "Prevention" can include a delay of onset, as well as a partial or complete block in progress of the disease or disease symptoms.

As used herein, "a naturally-processed peptide fragment" is a peptide fragment produced by proteolytic degradation of a protein in an antigen presenting cell of a mammal. As used herein, a "tumor antigen" is a molecule (e.g., a protein molecule) that is expressed by a tumor cell. Such a molecule can differ (e.g., by one or more amino acid residues where the molecule is a protein) from, or it can be identical to, its counterpart expressed in

normal cells. The tumor antigen is preferably not expressed by normal cells. Alternatively, it is expressed at a higher level (e.g., a two-fold, three-fold, five-fold, ten-fold, 20-fold, 40-fold, 100-fold, 500-fold, 1,000-fold, 5,000-fold, or 15,000-fold higher level) in a tumor cell than in the tumor cell's normal counterpart. Examples of tumor antigens include, without limitation, CEA, prostate specific antigen (PSA), MAGE (melanoma antigen) 1-4, 6, 11-12, A10, and C1, MUC (mucin) (e.g., MUC-1, MUC-2, etc.), tyrosinase, MART (melanoma antigen), Pmel 17(gp100), GnT-V intron V sequence (N-acetylglucoaminyltransferase V intron V sequence), Prostate Ca psm, PRAME (melanoma antigen), β-catenin, MUM-1-B (melanoma ubiquitous mutated gene product), GAGE (melanoma antigen) 1, BAGE (melanoma antigen) 2-10, c-ERB2 (Her2/neu), EBNA (Epstein-Barr Virus nuclear antigen) 1-6, gp75/TRP 1, human papilloma virus (HPV) E6 and E7, p53, lung resistance protein (LRP) Bc1-2, and Ki-67, RAGE, LAGE-1, CAG-3, DAM, NY-ESO- 1, CDK4, BRCA2, NY-LU- 1, NY-LU-7, NY-LU- 12, CASP8, RAS, KIAA-2-5, SCCs, p53, p73, CEA, Her 2/neu, Melan-A, TRP2, kallikrein, PAP, PSA, PT 1-1, B-catenin, Telomerase, FAK, cyclin D1 protein, NOEY2, EGF-R, SART- 1, CAPB, HPVE7, p 15, Folate receptor CDC27, PAGE- 1, and PAGE-4. Recognition of such a peptide by $CD8^+$ T cells of a mammal (e.g., a human patient) is indicative of the existence, or future onset, of cancer in the mammal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred

methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. Unless otherwise indicated, these materials and methods are illustrative only and are not intended to be limiting. All publications, patent applications, patents and other references mentioned herein are incorporated by reference.

Other features and advantages of the invention, e.g., methods of identifying peptides that activate $CD8^+$ T lymphocyte responses, will be apparent from the following description, from the drawings and from the claims.

This invention applies our knowledge of the mechanisms by which antigen is recognized by T cells, for example, to develop epitope-based immunomodulatory preparations directed towards TAAs. More specifically, this application communicates our discovery of specific epitope pharmaceutical compositions and methods of use in the prevention and treatment of cancer.

Upon development of appropriate technology, the use of epitope-based immunomodulatory preparations has several advantages over current vaccines, particularly when compared to the use of whole antigens in vaccine compositions. For example, immunosuppressive epitopes that may be present in whole antigens can be avoided with the use of epitope-based immunomodulatory preparations. Such immunosuppressive epitopes may, *e.g.*, correspond to immunodominant epitopes in whole antigens, which may be avoided by selecting peptide epitopes from non-dominant regions *(see, e.g., Disis et al., J. Immunol. 156:3151-3158, 1996)*.

An additional advantage of an epitope-based immunomodulatory preparations approach is the ability to combine selected epitopes and further, to modify the composition of the epitopes, achieving, for example, enhanced immunogenicity. Accordingly, the immune response can be modulated, as appropriate, for the target disease. Similar engineering of the response is not possible with traditional approaches.

Another major benefit of epitope-based immunomodulatory preparations is their safety. The possible pathological side effects caused by infectious agents or whole protein antigens, which might have their own intrinsic biological activity, is eliminated.

An epitope-based immunomodulatory preparation also provides the ability to direct and focus an immune response to multiple selected antigens from the same pathogen (a "pathogen" may be an infectious agent or a tumor-associated molecule). Thus, patient-by-patient variability in the immune response to a particular pathogen may be alleviated by inclusion of epitopes from multiple antigens from the pathogen in an immunomodulatory composition.

Furthermore, an epitope-based anti-tumor immunomodulatory preparation also provides the opportunity to combine epitopes derived from multiple tumor-associated molecules. This capability can therefore address the problem of tumor-to-tumor variability that arises when developing a broadly targeted anti-tumor immunomodulatory preparation for a given tumor type and can also reduce the likelihood of tumor escape due to antigen loss. For example, a breast cancer tumor in one patient may express a target TAA that differs from a breast cancer tumor in

another patient. Epitopes derived from multiple TAAs can be included in a polyepitope immunomodulatory preparation that will target both breast cancer tumors.

Brief Description of the Drawings

Fig. 1 is a MS/MS Spectrum of naturally processed YLWWVNNQSL (SEQ ID NO:1). Spectral labeling in Figs. 1-7 use the nomenclature described by Roepstorff and Fohlman (1988), Biomed. Mass. Spectrom. 11:601, and Biemann (1990), Meth. Enzymol. 193:866-867.

Fig. 2 is a MS/MS spectrum of naturally processed YLWWVNDQSL (SEQ ID NO:2)

Fig. 3 is a MS/MS spectrum of naturally processed YLWWVNGQSL (SEQ ID NO:3).

Fig. 4 is a MS/MS spectrum of naturally processed YLSGANLNL (SEQ ID NO:4). There were a number of background responses (marked by *) that were derived from a precursor ion that co-eluted chromatographically with YLSGANLNL (SEQ ID NO:4) and was closely related in m/z. These Such background could be only partially resolved chromatographically from YLSGANLNL (SEQ ID NO:4) but this did not prevent the identification of YLSGANLNL (SEQ ID NO:4) from the resultant composite mass spectrum.

Fig. 5 is a MS/MS spectrum of naturally processed ATVGIMIGV (SEQ ID NO:6).There were a number of background responses (marked by *) that were derived from a precursor ion that co-eluted chromatographically with ATVGIMIGV (SEQ ID NO:6) and was closely related in m/z. These Such background could be only partially resolved chromatographically from ATVGIMIGV (SEQ ID NO:6) but this

did not prevent the identification of ATVGIMIGV (SEQ ID NO:6) from the resultant composite mass spectrum.

Fig. 6 is a MS/MS spectrum of naturally processed IMIGVLVGV (SEQ ID NO:7).

Fig. 7 is a MS/MS spectrum of naturally processed GVLVGVALI (SEQ ID NO:8).

Fig. 8 is the primary sequence of CEA with the SEQ ID NO 1-5 highlighted. The c-terminal 16 amino acids contains SEQ ID NO. 5-8.

DETAILED DESCRIPTION

The peptide epitopes and corresponding nucleic acid compositions of the present invention are useful for stimulating an immune response to a TAA by stimulating the production of $CD8^+$ T lymphocyte responses. The peptide epitopes, which are derived directly or indirectly from native TAA protein amino acid sequences, are able to bind to HLA molecules and stimulate an immune response to the TAA. The complete sequence of the TAA proteins to be analyzed can be obtained from GenBank. Peptide epitopes and analogs thereof can also be readily determined from sequence information that may subsequently be discovered for heretofore unknown variants of particular TAAs, as will be clear from the disclosure provided below.

The present invention is based on the novel discovery that peptide epitopes from tumor cells can be isolated, identified and used to stimulate $CD8^+$ T cells capable of recognizing tumor cells in mammals (e.g. disease tissue in human patients). The TAA are degraded by proteolytic enzymes into peptide fragments. If any of these peptide fragments has, by virtue of its length and sequence, the ability to bind to one of the class I MHC molecules

expressed by the cell, it will do so in the endoplasmic reticulum. The resulting peptide-class I MHC molecular complex is then transported to the cell's cell membrane, where it becomes available for interaction with $CD8^+$ T cells bearing antigen-specific receptors that specifically recognize that particular peptide-class I MHC complex. By eluting peptides from class I MHC molecules isolated from these cells, a set of naturally processed peptides derived from the TAA, as well as from other polypeptides of intracellular or extracellular origin, is obtained. The peptides, which are specific to the particular types (isotypes and alleles) of class I MHC molecules expressed by the APC, are then chemically separated and their amino acid sequences determined. By comparison of the peptide amino acid sequences to the sequence of the TAA, it is possible to identify those which are derived from the TAA. Thus, the present invention includes a method of identifying peptide fragments that are naturally processed by APC and have intrinsic binding affinity for the relevant class I MHC molecule. The method can be invaluable for identifying peptides derived from a polypeptide suspected of being an antigen that activates $CD8^+$ T cells involved in either (a) the pathogenesis (pathology) of a disease, especially one in which susceptibility or protection is known to be associated with expression of a particular type of class I MHC molecule, or (b) prevention or reduction of the symptoms of a disease, especially one in which protection or a reduction in severity is associated with expression of a particular type of class I MHC molecule.

The described method ensures that the peptides identified are those that both (i) are naturally processed

in vivo by the cell, and (ii) become associated, in the cell, with the relevant class I MHC molecules.

Furthermore, the present method controls for class I MHC type, an important aspect essential to link any given peptide to a particular $CD8^+$ T cell-mediated disease in a given individual but especially important in disorders in which class I MHC type determines disease susceptibility or resistance.

Any naturally processed peptide with a sequence that corresponds to a fragment of the TAA, and which binds to a class I MHC molecule associated with the disease of interest, could be a peptide that activates $CD8^+$ T cells that exacerbate the disease or mediate immunity to it. To obtain confirmatory evidence of this possibility, test $CD8^+$ T cells from subjects expressing the relevant class I MHC molecules can be assayed for responsiveness to a peptide identified in accordance with the invention. A significant response of the test $CD8^+$ T cells and no or little response of the control $CD8^+$ T cells would indicate that the relevant peptide is involved either in the disease process (pathology of the disease) or in immunity to the disease. The cellular response phase of the method is designated "Epitope Verification" ("EV").

By applying the methods of the invention to the tumor antigen CEA, CEA-derived peptides were identified as epitopes that could be involved in the pathogenesis of cancer in human patients expressing the HLA-A2 class I MHC allele. Based on their amino acid sequences, these peptides fall into 5 domains. A consensus peptide corresponding to the binding region of each domain can be synthesized and tested for its ability to activate $CD8^+$

T cells from HLA-A2-expressing cancer patients or donors and recognizing tumor cells expressing both HLA-A2 and CEA.

Various strategies can be utilized to evaluate immunogenicity, including:

1) Evaluation of primary T cell cultures from normal individuals *(see, e.g.,* Wentworth, P. A. *et al., Mol. Immunol.* 32:603, 1995; Celis, E. *et al., Proc. Mad. Acad Sci.* USA 91:2105, 1994; Tsai, V. *et al., J Immunol.* 158:1796, 1997; Kawashima, 1. *et al., Human Immunol.* 59:1, 1998); This procedure involves the stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a test peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks. T cells specific for the peptide become activated during this time and are detected using, eg., a Elispot assay involving peptide sensitized target cells.

2) Immunization of HLA transgenic mice *(see, e.g.,* Wentworth, P. A. *et al., J Immunol.* 26:97, 1996; Wentworth, P. A, *et al., Int Immunol.* 8:651, 1996; Alexander, J. *et al., J Immunol.* 15 9:4753, 1997); In this method, peptides in incomplete Freund's adjuvant are administered subcutaneously to HLA transgenic mice. Several weeks following immunization, splenocytes are removed and cultured *in vitro* in the presence of test peptide for approximately one week. Peptide-specific T cells are detected using *e.g.,* an Elispot assay involving peptide sensitized target cells and target cells expressing endogenously generated antigen.

3) Demonstration of recall T cell responses from patients who have been effectively vaccinated or who have a tumor; (see, e.g., Rehermann, B. et al., J *Exp. Med.*

181:1047, 1995; Doolan, D. L. et al., *Immunity* 7:97, 1997; Bertoni, R. et al., J Clin. Invest. 100:503, 1997; Threlkeld, S. C. et al., J *Immunol.* 159:1648, 1997; Diepolder, H. M. et al., J Virol. 71:6011, 1997; Tsang et al., J. *Natl.* Cancer Inst. 87:982990, 1995; Disis et al., J *Immunol.* 156:3151-3158, 1996). In applying this strategy, recall responses are detected by culturing PBL from patients with cancer who have generated an immune response "naturally", or from patients who were vaccinated with tumor antigen immunomodulatory preparations. PBL from subjects are cultured in vitro for 1-2 weeks in the presence of test peptide plus antigen presenting cells (APC) to allow activation of "memory" T cells, as compared to "naive" T cells. At the end of the culture period, T cell activity is detected using assays for T cell activity including an Elispot assay involving peptide-sensitized targets, T cell proliferation, or lymphokine release.

The methods of the invention can be applied to identifying peptides involved in the pathogenesis of or protection from any of a wide range of diseases, especially those in which relative susceptibility or resistance has been associated with expression of a particular class I MHC allele, provided that the amino acid sequence (or partial amino acid sequence) of a suspect polypeptide antigen is available. Candidate diseases include, without limitation, infectious diseases (e.g., diseases caused by *Chlamydia trachomatis, Helicobacter pylori, Neisseria meningitidis, Mycobacterium leprae, M. tuberculosis,* Measles virus, hepatitis C virus, human immunodeficiency virus, and *Plasmodium falciparium*), cancer (e.g. melanoma, ovarian cancer, breast cancer, colon cancer and B cell lymphomas)

1. Methods of Identifying $CD8^+$ T Cell Activating Peptide Epitopes Derived From Polypeptide Antigens

The methods of the invention have two distinct phases. The first is identification of epitopes that were specifically derived from a tumor associated antigen (TAA) and the second is "Epitope Verification" (EV). The purpose of the first stage of the process is to identify the individual peptide fragments generated by the proteasome complex of the source cell from the candidate polypeptide TAA. Any peptides of the appropriate length (about 8 to 15 amino acid residues), and having specific binding affinity for a particular class I MHC molecule expressed by the target cell or tissue, will bind to that class I MHC molecule in the endoplasmic reticulum. At least some of these peptide-class I MHC molecular complexes then migrate to the cell membrane of the cell. The complexes (both cell-membrane associated and intracellular) are isolated from the tissue/cell source and the peptides eluted from the complexes. The eluted peptides are then separated, their amino acid sequences determined, and the sequences compared to that of the candidate polypeptide.

The described method can generally be applied to the analysis of peptides produced by any tissue/cell source expressing defined class I MHC molecules. As such, the method can be useful for basic research studies, e.g., studies aimed at identifying amino acid residues in a polypeptide that determine sites of "cutting" by the proteolytic antigen processing enzymes of any tissue/cell source. Alternatively, where the polypeptide is suspected of being an antigen that activates $CD8^+$ T cells which cause

or promote a particular disease or mediate protection from a disease, the described method can be used to identify disease-related or protective peptide epitopes derived from the polypeptide. This information would be useful for basic research into the etiology of the disease, or as a basis for development of diagnostics, therapeutics, or immunomodulatory preparations for the disease.

A peptide whose amino acid sequence matches that of a region of the candidate polypeptide is likely to be one that activates $CD8^+$ T cells involved in the pathogenesis of or immunity to the relevant disease. Such a peptide can be subjected to the EV procedure in which its ability to activate $CD8^+$ T cells is assayed. Those peptides that activate $CD8^+$ T cells are identified as peptides that can mediate protection from disease or its pathogenic symptoms.

Once such a peptide is identified, it can be synthesized in large amounts, by chemical or recombinant techniques, and used in diagnostic assays similar to the EV procedures listed below. Relevant peptides could be used singly or in combination. Alternatively, expression vectors encoding such a peptide or a combination of such peptides can be used to transfect or transduce appropriate APC (see below), and these can be used in similar diagnostic assays.

Furthermore, multimers (e.g., dimers, trimers, tetramers, pentamers, or hexamers) of a class I MHC molecule associated with a peptide defined by the method of the invention and conjugated to a detectable label (e.g., a fluorescent moiety, a radionuclide, or an enzyme that catalyzes a reaction resulting in a product that absorbs or emits light of a defined wavelength) can be used to

quantify T cells from a subject (e.g., a human patient) bearing cell surface receptors that are specific for such complexes. Relatively high numbers of such T cells are likely to be diagnostic of a relevant disease or an indication that the T cells are involved in immunity to the disease. In addition, continuous monitoring of a patient's relative numbers of multimer-binding T cells can be useful in tracking the course of a disease or the efficacy of therapy. Such assays have been developed using tetramers of class I MHC molecules associated with an HIV-1-derived or an influenza virus-derived peptide [Altman et al. (1996), Science 274:94-96; Ogg et al. (1998), Science 279:2103-2106]. Such complexes could be produced by chemical cross-linking of purified class I MHC molecules assembled in the presence of a peptide of interest or by modification of already established recombinant techniques for the production of class I MHC molecules containing a single defined peptide.

1.1 Epitope Identification Method

In the Epitope Identification Method, a sufficient number of cells are harvested (~1 x 10^8 to 1 x 10^{10} depending on the expression of the target tumor antigen and the class I MHC molecules), and the class I MHC molecules of interest are isolated by any one of various methods known in the art, e.g., immunoprecipitation. They may be isolated by affinity chromatography by the method of Urban et al. (1994), PNAS 91, 1534.

Peptides bound non-covalently to the isolated class I MHC molecules are then eluted from the latter. A variety of methods known in the art can be used, for example, the method of Chicz et al. (1992), Nature 358:764-768; Chicz

and Urban, Immunology Today 15:155-160; Urban et al, Critical Reviews in Immunology 17:387-397 or the novel solid phase extraction protocol as in Example 1.

The eluted peptides are separated by one of a variety of possible chromatographic methods, e.g., reverse phase chromatography. All the resulting fractions that contain peptides are then individually analyzed by mass spectrometry, using settings that do not fragment the peptides. The peptides corresponding to all the "peaks" obtained on the mass spectra can then be subjected to individual amino acid sequence analysis. The sequences of the individual peptides can be obtained by means known to those in the art. They can, for example, be obtained by LC/MS/MS, using instrument settings resulting in the fragmentation of the peptides into small fragments that are analyzed by the mass spectrometer. The amino acid sequences of the peptides are then compared to that of the TAA. Those with a sequence identical to a region of the TAA are candidates for EV.

Alternatively, other approaches for deriving the amino acid sequences of individual peptides can be utilized. These methods invoke a second dimension of peptide separation prior to mass spectrometric analysis. This is often achieved by coupling a separation technique such as reversed phase HPLC to a mass analyzer (such as but not limited to a quadrupole ion trap, linear ion trap, triple quadrupole instrument, magnetic sector, Fourier transform ion cyclotron resonance, quadrupole time-of-flight, or a hybrid of these analyzers) though an electrospray interface. Fractions can be analyzed in a data dependent mode of operation. In this mode, mass peaks are

dynamically and automatically selected for isolation and fragmentation to yield amino acid sequences. No prior knowledge of novel signals is required for this mode of peptide sequencing. Alternatively, fractions can be analyzed in a target dependent mode of operation. In this mode, mass peaks are targeted for isolation and fragmentation to yield amino acid sequences. Prior knowledge of ion signals is required for this mode of peptide sequencing.

1.2 EV

The EV procedure involves testing of peptides identified by the described epitope identification method for their ability to (a) bind the class I MHC from which they were eluted and (b) activate various $CD8^+$ T cell populations. Peptides with amino acid sequences identical to those identified by the described method are synthesized. The synthetic peptides are then tested for their ability to bind the class I MHC from which they were eluted and activate $CD8^+$ T cells from (a) test subjects expressing the class I MHC molecule of interest. In diseases (e.g., cancer or infectious diseases without an autoimmune component), a pattern of responsiveness would indicate that the relevant peptide is an epitope that activates $CD8^+$ T cells that can mediate immunity to the disease or, at least, a decrease in the symptoms of the disease.

$CD8^+$ T cell responses can be measured by a variety of *in vitro* methods known in the art. For example, whole peripheral blood mononuclear cells (PBMC) can be cultured with and without a candidate synthetic peptide and their cytokine production responses measured by, e.g., Elispot

assay. Cytokines include, without limitation, interleukin-2 (IL-2), IFN-γ, IL-4, IL-5, TNF-α, interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), GM-CSF, RANTES, MIP-1α, MIP-1β and transforming growth factor β (TGFβ). Assays to measure them include, without limitation, ELISA, ELISPOT and bio-assays in which cells responsive to the relevant cytokine are tested for responsiveness (e.g., proliferation) in the presence of a test sample. Alternatively, cytokine production by $CD8^+$ lymphocytes can be directly visualized by intracellular immunofluorescence staining and flow cytometry.

Once peptide epitopes associated with a particular disease have been identified, the EV described above can be used as a diagnostic test for the disease. Thus, lymphocytes from a subject suspected of having or being susceptible to the disease can be tested by any of the described methods for a $CD8^+$ T lymphocyte response to one or more (e.g., 2, 3, 4, 5, 6, 10, 15, or 20) appropriate peptides. If a significant $CD8^+$ T lymphocyte is detected, it is likely that the subject has or will develop the disease. The disease can be, for example, cancer and the peptides can be derived from, for example, CEA. Appropriate peptides can be, for example, any of those listed below (e.g., those with SEQ ID NOS:1-8).

As an alternative to the above-described EV, peptides identified by the epitope identification method can be tested for their ability to bind to an appropriate class I MHC molecule by methods known in the art using, for example, isolated class I MHC molecules or cells transfected with nucleic acid molecules encoding class I MHC molecules. One such method is described in Example 2.

These binding assays can also be used to test the ability of the peptides to bind to alternative class I MHC molecules, i.e., class I MHC molecules other than those from which they were eluted using the described method of the invention. Once such alternative class I MHC molecules are shown to bind the peptide(s), the diagnostic methods of the invention (using such peptides) and therapeutic methods of the invention can be applied to subjects expressing such alternative class I MHC molecules.

1.3 Diseases and their associations with class I MHC genes

The methods of the invention can be applied to the analysis of peptides involved in diseases associated with expression of defined class I MHC molecules and in which pathology or protection is due to the action of activated $CD8^+$ T cells. Such diseases include, without limitation, certain infectious diseases, cancer, and autoimmune diseases.

Melanoma cell-specific $CD8^+$ T cells, which may be involved in protective immune responses to malignant melanoma, recognize tyrosinase epitopes presented by HLA-A2 class I molecules.

1.4 Species

The methods of the invention can be applied to diseases with the described characteristics in a wide range of mammalian species, e.g., humans, non-human primates, horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, hamsters, rats, and mice. They will preferably be applied to diseases of humans.

1.5 Class I MHC Molecules

Class I MHC molecules have been identified in multiple mammalian species. In some of these species, expression of a particular class I MHC molecule has been associated with a particular $CD8^+$ T cell-mediated diseases (see above). In humans, for example, the class I MHC molecules are designated HLA-A, HLA-B, and HLA-C and in mice, H-2D, H-2K and H-2L. In all species, there are multiple alleles of each gene.

1.6 Tissue/cell sources

Tissue/cell sources that can be used for the epitope identification methods of the invention can be any mammalian cell that expresses class I MHC molecules on its surface, e.g., those listed above for use in EV (B lymphocytes, macrophages, monocytes, dendritic cells, and, in humans, T cells). Tissue/cell sources can also be tumor cells, e.g., B cell lymphoma cells or adenocarcinoma cells. It is also not required that the cells constitutively express class I MHC molecules. Class I MHC can be induced (*in vitro* or *in vivo*) (e.g., by IFN-γ) in such cells. Alternatively, immortalized lines of such cells can be used.

1.7 Polypeptide Antigens

Polypeptide antigens that can be used with the epitope identification methods can be those with a known amino acid sequence or those in which at least part of the amino acid sequence is known. They can be polypeptides that themselves are known or suspected to be involved in the disease process or immunity to the disease (e.g., CEA in cancer) or they can be derived from microbial organisms known or suspected to be involved in the disease process (e.g., *M. leprae* in leprosy). Examples of other polypeptide antigens include the core and viral coat proteins of viruses such as hepatitis C virus, the heat shock proteins of mycobacteria, and tyrosinase in melanoma. Furthermore, the polypeptide antigen can be the full-length protein or it can be a fragment of the protein known or suspected to be involved in the disease process.

2. Peptides

Peptides of the invention include peptides that bind to class I MHC molecules and activate $CD8^+$ T cells involved in a disease process or protection from a disease. The class I MHC molecule can be a class I MHC molecule that is associated with susceptibility or resistance to a disease. Diseases can be any of the diseases cited herein and the species from which the class I MHC molecules and/or peptides are obtained can be any of those cited herein. The class I MHC molecules are preferably human class I HLA molecules, i.e., A, B or C molecules. The peptides can be, for example, peptides that bind to HLA-A2 molecules. The polypeptides from which the peptides of the invention are derived can be any of those cited herein. The peptides generally are 8 to 15 (e.g., 9 to 15) amino acids in length.

The peptides can be derived, for example, from CEA and can bind to HLA-A2 molecules. The peptides can be, for example, any one of the following peptides: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends. Also included are peptides containing any of the above sequences, plus 1-15 residues on either or both ends.

In addition, peptides identified as being associated with any of the diseases listed herein (e.g., cancer such as colon cancer or any of the infectious diseases recited herein) can be used to activate lymphocytes (e.g., CD8+ lymphocytes) that cause, directly or indirectly, the death of pathogenic target cells such as cancer cells or

pathogen-infected cells. In addition, peptidomimetic forms of the peptides can be produced by methods known in the art. The peptides can be fragments of any the polypeptides disclosed herein, e.g., CEA or PSA. They can be, for example, those with SEQ ID NOS:1-8.

The CEA peptides of the invention (e.g., those with SEQ ID NOS:1-8), can be used for purposes other than therapy. They can be used, for example, in diagnostic assays and for methods of screening for reagents that bind to complexes of class I MHC molecules and CEA peptides (see below).

The peptides can be prepared using the described epitope identification methodologies. Smaller peptides (fewer than 50 amino acids long) can also be conveniently synthesized by standard chemical means. In addition, both polypeptides and peptides can be produced by standard *in vitro* recombinant nucleic acid techniques, and *in vivo* transgenesis using the nucleotide sequences encoding the appropriate polypeptides, peptides or APL. Methods well known to those skilled in the art can be used to construct expression vectors containing relevant coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook et al., Molecular Cloning: A Laboratory Manual [Cold Spring Harbor Laboratory, N.Y., 1989], and Ausubel et al., Current Protocols in Molecular Biology, [Green Publishing Associates and Wiley Interscience, N.Y., 1989].

The invention also features isolated nucleic acid molecules encoding the peptides of the invention. These nucleic acid molecules can be cDNA, genomic DNA, synthetic DNA, oligonucleotides, ribozymes or RNA, and can be

double-stranded or single-stranded (*i.e.*, either a sense or an antisense strand). Segments of these molecules are also considered within the scope of the invention, and can be produced, for example, by the polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription. Preferably, the nucleic acid molecules encode peptides that, regardless of length, are soluble under normal physiological conditions.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide (for example, the peptides with SEQ ID NOS:1-8). In addition, these nucleic acid molecules are not limited to coding sequences, e.g., they can include some or all of the non-coding sequences that lie upstream or downstream from a coding sequence.

The nucleic acid molecules of the invention can be synthesized (for example, by phosphoramidite-based synthesis) or obtained from a biological cell, such as the cell of a mammal. Thus, the nucleic acids can be those of a human, non-human primate (e.g., monkey) mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, dog, or cat.

In addition, the isolated nucleic acid molecules of the invention encompass segments that are not found as such in the natural state. Thus, the invention encompasses recombinant nucleic acid molecules, (for example, isolated nucleic acid molecules encoding any of the peptides described herein) incorporated into a vector (for example,

a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location).

Certain nucleic acid molecules of the invention are antisense molecules or are transcribed into antisense molecules. These can be used, for example, to down-regulate translation of CEA mRNA within a cell.

Techniques associated with detection or regulation of genes are well known to skilled artisans and such techniques can be used to diagnose and/or treat disorders associated with aberrant CEA expression, e.g., colon cancer. Hybridization can be used as a measure of homology between two nucleic acid sequences. Thus a nucleic acid encoding peptide of the invention (e.g., CEA peptides such as the peptide with SEQ ID NOs: 1-8), or a portion of such a nucleic acid, can be used as hybridization probe according to standard hybridization techniques. The hybridization of a CEA peptide probe to DNA or RNA from a test source (e.g., a mammalian cell) is an indication of the presence of CEA DNA or RNA, respectively, in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by one or more washes in 1 X SSC, 0.1% SDS at 50-60°C. Highly stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

The invention also encompasses: (a) vectors that contain any of the foregoing peptide coding sequences and/or their complements (that is, "antisense" sequence); (b) expression vectors that contain any of the foregoing peptide coding sequences operatively associated with any transcriptional/translational regulatory element necessary to direct expression of the coding sequences; (c) expression vectors containing, in addition to sequences encoding a peptide of the invention, nucleic acid sequences that are unrelated to nucleic acid sequences encoding the peptide of the invention, such as nucleic acid sequences encoding a reporter, marker, or a signal peptide (e.g., a heterologous signal peptide); and (d) genetically engineered host cells that contain any of the foregoing expression vectors and thereby express the nucleic acid molecules of the invention.

Where the nucleic acids form part of a hybrid gene encoding additional polypeptide sequences, for example, sequences that function as a marker or reporter, markers or reporter genes include β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, $G418^r$), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β-galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, additional sequences that can serve the function of a marker or reporter. Generally, the hybrid polypeptide will include a first portion and a

second portion, the first portion being a peptide of the invention (e.g., a peptide with any of SEQ ID NOS:1-8) and the second portion being, for example, a reporter described above or an immunoglobulin constant region.

A variety of host-expression vector systems can be used to express the peptides and polypeptides. Such host-expression systems represent vehicles by which the polypeptides of interest can be produced and subsequently purified, but also represent cells that can, when transformed or transfected with the appropriate nucleotide coding sequences, produce the relevant peptide or polypeptide *in situ*. These include, but are not limited to, microorganisms such as bacteria, e.g., *E. coli* or *B. subtilis*, transformed with recombinant bacteriophage nucleic acid, plasmid or cosmid nucleic acid expression vectors containing polypeptide coding sequences; yeast, e.g., *Saccharomyces* or *Pichia*, transformed with recombinant yeast expression vectors containing the appropriate coding sequences; insect cell systems infected with recombinant virus expression vectors, e.g., baculovirus; plant cell systems infected with recombinant virus expression vectors, e.g., cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV), or transformed with recombinant plasmid expression vectors, e.g., Ti plasmids, containing the appropriate coding sequences; or mammalian cell systems, e.g., COS, CHO, BHK, 293 or 3T3, harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells, e.g., metallothionein promoter, or from mammalian viruses, e.g., the adenovirus late promoter or the vaccinia virus 7.5K promoter.

The following examples are meant to illustrate, not limit, the invention.

Example 1. Identification of Class I MHC Binding CEA Peptide Epitopes

Materials and Methods

Carcinoma cell lines expressing CEA and HLA-A2. The carcinoma cell lines, KATO III, LS180, LS174T, and SW480, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained under the culture conditions recommended by the supplier. The cell lines were propagated in 2 L roller bottles to a density of ~10^6 cells/mL in RPMI 1640 medium supplemented with glutamine, penicillin/streptomycin and 10% fetal calf serum (FCS). All cell lines were tested for CEA surface expression using the murine mAb COL-1 (specific for human CEA) by FACS and for relative quantification by Western blot analysis. CEA expression was verified on all cell lines. Genotype analysis of each cell line confirmed HLA-A*0201 expression. Surface expression of HLA-A2 was verified using the murine mAb BB7.2.

HLA class I purification. Cells were harvested and pelleted by centrifugation. The cell pellets were weighed to determine the cellular mass and then frozen at -80°C prior to lysis. Each cell pellet was resuspended in lysis buffer (2 ml per gram of pellet) containing 1% CHAPS, 500 mM NaCl, 20 mM Tris-OH pH 8.0, in Milli-Q™ reverse osmosis quality (about 18.2 μΩ) water containing freshly added protease inhibitors (100 μM iodoacetamide, 8 μg/ml aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 5 mM EDTA, 0.04 % sodium azide, 1 mM PMSF), and the cells were

lysed by gentle agitation for 1 hr at 4°C on a rotor table. The resulting cell lysate was sedimented in an ultra-centrifuge for 1 hr at 230,000 x g at 4°C (37,500 RPM on a SW41 TI rotor). The insoluble material was removed by sedimentation at 175,000 x g for 2 hours, and the soluble supernatant fraction used for subsequent HLA purification. Multi-modal protein purification using HPLC columns was achieved by coupling the chromatographic sorbents in series with automated switching valves that direct the class I HLA-peptide complex containing effluent to subsequent columns in the sequences. The first three coupled columns were connected directly in series and acted together as a single pre-clearing column using high strength large throughpore perfusion sorbents (6000-8000 Å throughpores and 500-1000 Å diffusive pores, 50 µm) coated and crosslinked with a hydrophilic stationary phase and covalently conjugated with Protein A as the sorbent. These columns were designed to remove those proteins that non-specifically bind to the sorbents. Column 1 contained unmodified Protein A sorbent, column 2 contained Protein A coated with normal mouse serum, and column 3 was Protein A coated with bovine serum. The pre-clearing columns were followed by an immunoaffinity column of Protein A coupled with mAb specific for a non-polymorphic determinant on HLA-A2 molecules (A-BB7.2). After passing the lysate through the immunoaffinity column, the column was extensively washed with 25 column volumes of 0.1 % CHAPS/500 mM NaCl/0.05 % sodium azide/20 mM Tris-OH pH 8.0, followed by 25 column volumes of 0.1 % DOC/20 mM Mops/280 mM NaCl/0.05 % sodium azide pH 8.0, and finally 50 column volumes of 0.1 % DOC/0.05 % sodium azide/10 mM Tris-

OH pH 8.0. The HLA-A2-peptide complexes were eluted from the immunoaffinity support using 3.5 column volumes of 50 mM carbonate/0.1% DOC/0.05% NaN_3 at pH 11.5.

Peptide Analysis

The HLA class I protein samples can be concentrated by various methods. In one such method, the HLA class I protein sample eluted from the immunoaffinity column was concentrated to 100 µl using an ultrafiltration device (Amicon Centricon 3) prior to peptide extraction. Naturally processed peptide mixtures were acid eluted from HLA class I molecules by adding 800 µl 10 % acetic acid and incubating for 15 minutes at 70°C, as previously described [Chicz et al. (1993), J. Exp. Med. 178:24-47]. The peptides were separated from the remaining HLA protein by ultrafiltration with an Amicon Centricon 3™ device. The "flow-through" fraction containing the acid-extracted peptides was concentrated on a Savant SpeedVac™ centrifugal vacuum concentrator to a volume of approximately 20-100 µl and stored at -80°C. The acid-extracted peptide mixtures were then separated by reverse phase chromatography as previously described [Chicz et al. (1993), supra] but with minor modifications. Briefly, peptide solutions were preconcentrated by trapping the peptides using a small bed (0.5 - 3.0 µL bed volume) of polymeric reversed phase support. This also facilitates removal of hydrophilic contaminants that may be in the sample by washing the trap with a suitable aqueous solution (e.g., the buffers used for the chromatographic separation of the isolated peptides). Subsequently, peptides were back-flushed from the trapping phase to a microbore C18 column (1.0 x 250 mm; Vydac, Hesperia, CA), and peptide fractionation was

performed with a gradient of conventional solvents (containing water, acetonitrile and a suitable ion pair reagent) at a flow rate of 50 µl/minute. The column effluent was collected and stored at -20°C prior to analysis by mass spectrometry.

An alternative approach was to concentrate HLA class I molecule samples and extract the associated peptides using solid phase extraction (SPE). In this method, the HLA class I molecule samples were concentrated using a protein capture column that contained a suitable HPLC stationary phase. Appropriate HPLC stationary phases include a reversed phase (preferably a silica based C4, C8, C18 phase or a polymeric C4, C8 or C18-like reversed phase). However, a cation or anion exchange resin (including both strong, weak and mixed bed ion exchange phases) or other substrates that exhibit high affinity for protein complexes while enabling the elution of the HLA class I peptides, are equally suitable. Suitable dimensions of the protein capture column are 1-4.6 mm internal diameter and 2-5 cm long. Once the protein was bound to the capture column, the column was washed with suitable solvents (e.g., an aqueous buffer containing 5-25 mM Tris base at pH 7.5-8.5 followed by an aqueous solution of trifluroacetic acid (0.05-0.2% v/v)) to remove hydrophilic contaminants. The HLA class I molecule-peptide complexes were next disrupted by the action of a suitable solvent that also facilitated the elution of peptides from the protein capture column while leaving proteins adsorb on the stationary phase. An appropriate solvent for this purpose is a mixture of acetonitrile (5-25% v/v), and TFA (0.05-5% v/v) in water. The eluted peptides were collected

in a suitable container (e.g., an Eppendorf™ microfuge tube) and concentrated using a Savant Speedvac™ centrifugal vacuum concentrator. This step lowered the acetonitrile concentration and thereby permitted peptide fractionation by reverse phase chromatography (as described above). All peptide samples were stored at -20°C prior to analysis by mass spectrometry. Alternatively, the eluted peptides were mixed post-elution from the protein capture column with an aqueous solution of TFA (typically 0.05-0.2% TFA in water) in order to lower the acetonitrile concentration and thereby permit peptide adsorption onto a peptide capture column. The peptide capture column contained an HPLC phase (e.g., a reversed phase resin such as C18 or polymeric equivalent or an ion exchange resin or other suitable phase that exhibits high affinity towards peptides). When the peptide capture column contained an ion exchange resin (such as a strong cation exchanger) no solvent manipulation was required, as such phases efficiently adsorb peptides by charge, and or hydrophilic mechanisms that are not disrupted by relatively high levels of organic solvents. Suitable dimensions of the peptide capture column are 0.5-4.6 mm internal diameter and 1-5 cm long. Following the adsorption of peptides, the peptide capture column was washed with a suitable solvent (e.g., 0-5% acetonitrile, 0.05-0.2% TFA in water) prior to peptide fractionation by reversed phase chromatography (as described above). All peptide samples were stored at -20°C prior to analysis by mass spectrometry.

An automated microcapillary liquid chromatography-mass spectroscopy (LC-MS) approach with either targeted or data dependent collision-assisted dissociation (CAD) was used to

sequence low levels of naturally processed HLA associated peptides. Peptide fractions separated by reversed phase chromatography were diluted to aid handling and permit second dimension reversed phase separations. Typically, 1-5 µL aliquots of each peptide fraction were diluted to a final volume of 5-20 µL with a suitable solvent. A suitable solvent is one that is predominantly aqueous, that contains a low concentration 1-10 % by volume) of acetonitrile and an ion pair reagent such as TFA (at 0.1 - 1.0% by volume). Each diluted peptide solution was then concentrated by trapping peptides in a small bed (0.5 - 1.0 µL bed volume) of polymeric reversed phase support. This step also facilitated removal of hydrophilic contaminants by washing the trap with a suitable aqueous solution (e.g., mobile phase A as used in the chromatographic separation of isolated peptides). The peptides were then back flushed from the trapping phase onto the microcapillary (with an inner diameter of 75 µm and packed with 3-10 cm of 1-7 µm 100-300 Å C_{18} or non-porous material) and separation was developed using a non-linear gradient of conventional mobile phases for peptide separations (typically combinations of water and acetonitrile containing a suitable ion pair reagent). A mobile phase flow rate through the capillary column of 0.15-1.0 µL/min was achieved by splitting the flow from the pumps and using a backpressure regulator on the solvent waste line. Peptide detection was by µ-electrospray mass spectrometry. The voltage necessary to drive the electrospray was applied at the head of the microcapillary column (using a liquid junction interface) and peptides were electrosprayed into the mass analyzer directly as they eluted from the column.

CAD experiments were either predetermined to conduct specific target analyses or triggered in a data dependent mode, using ions that were more abundant than a user-set threshold. Dynamic exclusion was used in conjunction with data dependent analyses to ensure maximum peptide coverage (i.e., minor responses were analyzed by CAD following a user-determined number of CAD experiments of a single peptide response) by writing an exclusion list during assay progression so that a given ion will not be analyzed by multiple CAD experiments. The time that a given ion resides on the exclusion list was dependent upon the quality of the chromatographic separations. This time is determined experimentally. In this way, separated isobaric responses may be analyzed. Peptide sequencing sensitivities greater than 100 attomoles were achieved using this method. Alternatively, peptide fractions were reduced using microchemistries that convert oxidized methionine and cysteine containing peptides to their native forms. This chemistry requires the exchange of the acidic solution of reversed phase peptide fractions to be exchanged for a buffer of pH 8-9. This is conveniently achieved by concentrating each peptide fraction to dryness, and re-dissolving the peptide-containing residue in an ammonium bicarbonate buffer (100 mM in water at pH 8.2). The ammonium bicarbonate solution also contains a suitable reducing agent (such as 2-mercaptoethanol or dithiothreitol (DTT) or a combination of both reagents). The reducing agent is typically used at a concentration of 50 - 300 pmol/μL, and 5-20 μL of the reagent is sufficient for quantitative reduction of the peptide mixture. The reaction is typically performed at 37°C for 30 to 60 mins.

Subsequently the peptide fraction is reduced to dryness and re-dissolved in a solvent (as described above) that is suitable for peptide analysis by microcapillary LC/MS/MS. Reduced peptide fractions are analyzed as described for their non-reduced counterparts.

Results

Each preparation of tumor cells was grown to a density of ~10^6 cells/mL and harvested by sedimentation from 2 L roller bottles containing growth medium. Cell growth and viability varied among the cell lines. The three adherent cell lines were more difficult to consistently maintain in large scale culture and thus only SW480 was produced above the 20g threshold. The total amount of cells prepared were as follows: KATO III, 24.4g; SW480, 26.3g; LS174T, 17.6g; LS180, 12.3 g.

Protein purification was accomplished by immunoaffinity chromatography as described above. The HLA-A2 yields from the Protein-A-BB7.2 column for each preparation varied depending on the constituent expression levels of the target cell line and the amount of cell pellet processed. The HLA-A2 protein yields for the preparations listed above were as follows: KATO III, 40 ug; SW480, 19 ug; LS174T, 6 ug; and LS180, 2 ug.

Peptides were eluted from each HLA-A2 protein preparation and separated by RP-HPLC. The intact repertoire of peptides was separated into 100 fractions. RP-HPLC analysis was highly reproducible. Each fraction was analyzed by LC/MS/MS as described above in either a data dependent or targeted mass mode of operation.

Approximately 1050 analyses, including multiple analyses on the same sample, were conducted using LC/MS/MS

on the combined set of separated fractions. The overall analysis included both LC/MS and LC/MS/MS spectra collection. The initial preparation used for screening purposes was from the gastric carcinoma cell line, KATO III. Both triple play (MS, Zoom scan and MS/MS) and multiple MS/MS (MS, followed by three MS/MS scans) approaches were used to detect and identify novel HLA-A2 presented CEA epitopes from Kato III. Ions were automatically selected for MS/MS fragmentation during data acquisition as they exceeded an intensity threshold. Improved dynamic range was achieved by automatically placing precursor masses on an exclusion list for a period of time after their MS/MS spectrum was collected. Acquired MS/MS spectra were searched and analyzed using sequence analysis software and a CEA specific protein database. Peptides identified by this approach were subsequently targeted in the other carcinoma cell lines using LC/MS/MS.

Five CEA peptides (SEQ ID NOS:1-4 and 7)were originally identified and sequence verified (Figs. 1-4 and 6) from the KATO III cell line. The remaining two CEA peptides (SEQ ID NOS:6 and 8)were originally identified and sequence verified (Figs. 5 and 7) from LS180. The yields of individual CEA peptide epitopes varied during biochemical isolation and identification between the four carcinoma cell lines. Nevertheless, multiple epitopes were identified in each of the four carcinoma cell lines (Table 1).

Table 1: Summary of CEA epitope library identified from individual carcinoma cell lines (- denotes not detected, +\- denotes product ions consistent with sequence, but of

low abundance and + denotes epitope detection and approximate level).

Identified Peptide	KATO III	SW480	LS174T	LS180
SEQ ID NO:1	+	-	-	-
SEQ ID NO:2	++++			
SEQ ID NO:3	+++++	-	-	-
SEQ ID NO:4	+\-	-	-	+
SEQ ID NO:6	-	-	-	+\-
SEQ ID NO:7	+++	+++	+	+++
SEQ ID NO:8	-	+	+/-	++

Thus, the described method applied to the analysis of peptides produced by natural processing of CEA identified seven peptides that are associated with HLA-A2. This knowledge provides the basis for the development of therapeutic and/or prophylactic agents against CEA associated cancers, e.g., colon cancer. It is expected that analogous methodologies can be similarly successful in identifying other class I MHC-restricted tumor antigen peptides that activate $CD8^+$ T cells and are involved in the $CD8^+$ T lymphocyte-mediated pathogenesis of other diseases (see above).

Epitope Verification (EV):

Because tissue specific and developmental TAAs are expressed on normal tissue at least at some point in time or location within the body, it may be expected that T cells to them, particularly dominant epitopes, are eliminated during immunological surveillance and that tolerance is induced. However, $CD8^+$ T lymphocyte responses

to tumor epitopes in both normal donors and cancer patient has been detected, which may indicate that tolerance is incomplete *(see, e.g.,* Kawashima *et al., Hum.* Immunol. 59.1, 1998; Tsang, *J. Natl. Cancer Inst.* 87:82-90,1995; Rongcun *et al., J. Immunol. 163:1037, 1999). Thus,* immune tolerance does not completely eliminate or inactivate $CD8^+$ T lymphocyte precursors capable of recognizing high affinity HLA class I binding peptides.

Once HLA binding peptides are identified, they can be tested for the ability to elicit a T-cell response. Briefly, peptides comprising epitopes from a particular antigen are synthesized and tested for their ability to bind to the appropriate HLA proteins. These assays may involve evaluating the binding of a peptide of the invention to purified HLA class I molecules in relation to the binding of a radioiodinated reference peptide. Alternatively, cells expressing empty class I molecules *(i.e.* lacking peptide therein) may be evaluated for peptide binding by immunofluorescent staining and flow microfluorimetry. Other assays that may be used to evaluate peptide binding include peptide-dependent class I assembly assays and/or the inhibition of $CD8^+$ T lymphocyte recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for $CD8^+$ T lymphocytes derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or *in vivo* $CD8^+$ T lymphocyte responses that can give rise to populations capable of reacting with selected target cells associated with a disease.

Conventional assays utilized to detect T cell responses include proliferation assays, lymphokine secretion assays, direct cytotoxicity assays, and limiting dilution assays. For example, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce $CD8^+$ T lymphocyte responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells. Alternatively, mutant non-human mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides and that have been transfected with the appropriate human class I gene, may be used to test for the capacity of the peptide to induce *in vitro* primary $CD8^+$ T lymphocyte responses.

Peripheral blood mononuclear cells (PBMCs) may be used as the responder cell source of $CD8^+$ T lymphocyte precursors, The appropriate antigen-presenting cells are incubated with peptide, after which the peptide-loaded antigen-presenting cells are then incubated with the responder cell population under optimized culture conditions. Positive $CD8^+$ T lymphocyte activation can be determined by assaying the culture for the presence of $CD8^+$ T lymphocytes that kill radio-labeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed forms of the antigen from which the peptide sequence was derived.

More recently, a method has been devised which allows direct quantification of antigen-specific T cells by staining with Fluorescein-labelled HLA tetrameric complexes (Altman, J. D. *et al.*, *Proc. Natl. Acad Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996). Other

relatively recent technical developments include staining for intracellular lymphokines, and interferon- release assays or ELISPOT assays. Tetramer staining, intracellular lymphokine staining and ELISPOT assays all appear to be at least 10-fold more sensitive than more conventional assays (Lalvani, A. *et al, J Exp. Med* 186:859, 1997; Dunbar, P. R. *et al.*, Curr. Biol. 8:413, 1998; Murali-Krishna, K. *et al., Immunity* 8: 177, 1998).

Alternatively, immunization of HLA transgenic mice can be used to determine immunogenicity of peptide epitopes. Several transgenic mouse models including mice with human HLA-A2 and B7 alleles have been characterized and others (*e.g.*, transgenic mice for HLA-A 1, -A3, -A11 and -A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed. Additional transgenic mouse models with other HLA alleles may be generated as necessary. Mice may be immunized with peptides emulsified in Incomplete Freund's Adjuvant and the resulting T cells tested for their capacity to recognize peptide-pulsed target cells and target cells transfected with appropriate genes. $CD8^+$ T lymphocyte responses may be analyzed using cytotoxicity assays described above.

To confirm that peptide epitopes identified are relevant to cancer (i.e., that they are recognized by $CD8^+$ T cells in the context of cancer), T cell recognition assays can be carried out using synthetic peptides having amino acid sequences based upon the sequence of the peptides identified by mass spectrometry to be derived from CEA (e.g., the peptides with SEQ ID NOS:1-4 and 6-8). Peptides can be synthesized using Fmoc chemistry and purified by RP-HPLC. The amino acid sequences and purity of greater than

90% for all the synthetic peptides can be confirmed by MALDI-MS and analytical HPLC. *In vitro* immunizations were performed with human lymphocytes from multiple leucopheresed healthy HLA-A2 donors. Peripheral blood mononuclear cells were obtained after a Ficoll-Hypaque gradient sedimentation. Effector cells were stimulated for 3 cycles at 7 days intervals. The peripheral lymphocytes were stimulated in the first cycle with dendritic cells (DC) and thereafter with peptide-pulsed adherent APC for the second and third cycle. DCs were pulsed 3 hours with 10ug/mL peptide in D-PBS, 1% BSA and 3ug/mL β-2m in a 24 well tissue plate and further cultured with peripheral blood lymphocytes (PBMC) ($2x10^6$/well). Cultures were restimulated with pulsed adherent cells on day 7 and day 14. IL-10 (10ng/mL) was added to the cultures 24 hours after each stimulation. IL-2 (10 IU/mL) was added to the cultures on days 9 and 16 and assays were performed day 21. HBV polymerase 455-463 (GLSRYVARL) peptide was used as the positive control.

T cell responses to CEA were analyzed by a IFN-gamma ELISPOT assay using a commercial IFN-gamma ELISPOT assay kit according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Briefly, effector cells were plated in 96 well plates in duplicate at $1x10^5$ cells/well. The cultures were stimulated with $1x10^5$ tumor target cells/well or T2 cells pulsed with 10ug/mL peptide. Each well of the 96-well hydrophobic PVDF membrane backed plate was previously absorbed with anti-IFN-γ monoclonal antibody (mAb) and blocked with 10% FCS for twenty minutes followed by an 24 hour incubation at 37°C in 5% CO_2. After which, each well

was washed four times and incubated overnight at 4°C with a biotinylated non-competing anti-IFN-γ mAb. Wells were washed three times, incubated for two hours at room temperature with streptavidin alkaline-phosphatase, washed again three times and developed with a thirty-minute incubation with BCIP/NBT and washed extensively with distilled water. IFN-γ^+ secreting cells (spots) were enumerated on an automated ELISPOT reader system with KS ELISPOT Software 4.2 (Zellnet Consulting, Inc., New York, NY).

Data obtained from the above EV analysis confirm that the peptides identified by the defined method are recognized specifically by $CD8^+$ T lymphocytes from five HLA-A2 expressing donors capable of targeting cancer cells expressing both HLA-A2 and CEA (Table 2).

Table 2: Summary of immunological verification for HLA-A2 presented CEA epitope library from five HLA-A2 donors (nt denotes not tested, (-) denotes not detected, and (+) denotes epitope detection and approximate level).

CEA epitope	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
SEQ ID NO:1	nt	nt	++	++++	++
SEQ ID NO:2	nt	nt	nt	++	nt
SEQ ID NO:3	nt	nt	++	++++	+
SEQ ID NO:4	-	++++	++	++	nt
SEQ ID NO:6	nt	nt	-	+	-
SEQ ID NO:7	+++	++++	++	++	nt
SEQ ID NO:8	++	+	-	++	nt

Example 2. Binding of peptides to isolated HLA-A2 molecules

The following example of peptide binding to HLA molecules demonstrates quantification of binding affinities of HLA class I peptides.

HLA class I binding assays using purified HLA-A2 molecules were performed in accordance with disclosed protocols (e.g., Sidney *et al.*, *Current Protocols* in Immunology 18.3.1 (1998); Sidney, *et al.*, J. Immunol. 154:247 (1995); Sette, *et al.*, *Mol. Immunol. 31:813* (1994)). Briefly, recombinant HLA-A2 molecules (5 to 500nM) were incubated with the ^{125}I radiolabeled HBV core 18-27 (FLPSDYFPSV) reference peptide and beta-2-microglobulin. The mixture was allowed to fold over a 48 hr incubation period. Stable HLA peptide complexes were separated from free peptide by size exclusion chromatography and the fraction of peptide bound was determined. Typically, in preliminary experiments, the HLA-A2 preparation was titered in the presence of fixed amounts of radiolabeled peptides to determine the concentration of HLA molecules necessary to bind 10-20% of the total radioactivity. All subsequent inhibition and direct binding assays were performed using these HLA concentrations.

Under these conditions the measured IC50 values are reasonable approximations of the true K_D values. Peptide inhibitors are typically tested at concentrations ranging from 120 ~g/ml to 1.2 ng/ml, and are tested in two to four completely independent experiments. To allow comparison of the data obtained in different experiments, a relative binding figure is calculated for each peptide by dividing the IC_{50} of a positive control for inhibition by the IC_{50} for each tested peptide (typically unlabeled versions of the radiolabeled probe peptide).

Peptides corresponding to the identified CEA epitopes were synthesized and tested in peptide binding experiments measuring the IC50 compared to a known HLA-A2 binding peptide. The results of the peptide binding assay are listed in Table 3. All seven identified CEA epitopes were capable of specific binding to HLA-A2. Five of the seven eptiopes bound with relatively high affinity with the remaining two epitopes representing weak binding affinity.

Table 3: IC50 results for CEA epitopes.

CEA epitope	IC50 (nM)
SEQ ID NO:1	22
SEQ ID NO:2	> 1000
SEQ ID NO:3	180
SEQ ID NO:4	81
SEQ ID NO:6	56
SEQ ID NO:7	27
SEQ ID NO:8	1122

Other Embodiments

The invention also features the following embodiments.

Methods of Use

Diagnostic Agents and for Evaluating Immune Responses

In one embodiment of the invention, HLA class I peptides as described herein are used as reagents to evaluate an immune response. The immune response to be evaluated is induced by using as an immunogen any agent that may result in the production of antigen-specific $CD8^+$ T lymphocytes that recognize and bind to the peptide epitope(s) to be employed as the reagent. The peptide reagent need not be used as the immunogen. Assay systems

that are used for such an analysis include relatively recent technical developments such as tetramers, staining for intracellular lymphokines and interferon release assays, or ELISPOT assays.

For example, peptides of the invention are used in tetramer staining assays to assess peripheral blood mononuclear cells for the presence of antigen-specific $CD8^+$ T lymphocytes following exposure to a tumor cell antigen or an immunogen. The HLA-tetrameric complex is used to directly visualize antigen-specific $CD8^+$ T lymphocytes *(see, e.g.,* Ogg *et al., Science* 279:2103-2106, 1998; and Altman *et al., Science* 174:94-96, 1996) and determine the frequency of the antigen-specific $CD8^+$ T lymphocyte population in a sample of peripheral blood mononuclear cells. A tetramer reagent using a peptide of the invention is generated as follows: A peptide that binds to an HLA molecule is refolded in the presence of the corresponding HLA heavy chain and β_2-microglobulin to generate a trimolecular complex. The complex is biotinylated at the carboxyl terminal end of the heavy chain at a site that was previously engineered into the protein. Tetramer formation is then induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to stain antigen-specific cells. The cells can then be identified, for example, by flow cytometry. Such an, analysis may be used for diagnostic or prognostic purposes. Cells identified by the procedure can also be used for therapeutic purposes.

Peptides of the invention are also used as reagents to evaluate immune recall responses *(see, e.g.,* Bertoni *et al., J. Clin. Invest.* 100:503-513, 1997 and Penna *et al., J*

Exp. Med. 174:1565-1570, 1991). For example, patient PBMC samples from individuals with cancer are analyzed for the presence of antigen-specific $CD8^+$ T lymphocytes using specific peptides. A blood sample containing mononuclear cells can be evaluated by cultivating the PBMCs and stimulating the cells with a peptide of the invention. After an appropriate cultivation period, the expanded cell population can be analyzed, for example, for $CD8^+$ T lymphocytes or for $CD4^+$ T lymphocyte activity.

The peptides are also used as reagents to evaluate the efficacy of an immunomodulatory preparation. PBMCs obtained from a patient vaccinated with an immunogen are analyzed using, for example, either of the methods described above. The patient is HLA typed, and peptide epitope reagents that recognize the allele-specific molecules present in that patient are selected for the analysis. The immunogenicity of the immunomodulatory preparation is indicated by the presence of epitope-specific $CD8^+$ T lymphocytes in the PBMC sample.

Immunomodulatory Compositions

Immunomodulatory preparations that contain an effective amount of one or more peptides a described herein are further embodiments of the invention. Once appropriately immunogenic epitopes have been defined, they can be sorted and delivered by various means, herein referred to as "immunomodulatory" compositions. Such immunomodulatory compositions can include, for example, peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres *(see, e.g.,* Eldridge, *et*

al., Mol. Immunol. 28:287-294, 1991: *Alonso et al., Vaccine* 12:299-306, 1994, Jones *et al., Vaccine* 13:675-681, 1995), peptide compositions contained in immune stimulating complexes (ISCOMS) (see, e.g., Takahashi *et al., Nature* 344:873-875, 1990; Hu *et al., Clin Exp Immunol.* 113:235-243, 1998), multiple antigen peptide systems (MAPs) *(see e.g.,* Tam, J. P., *Proc. Natl. Acad. Sci. USA.* 85:5409-5413, 1988; Tam, J.P., *J Immunol. Methods* 196:17-32, 1996), peptides formulated as multivalent peptides; peptides for use in ballistic delivery systems, typically crystallized peptides, viral delivery vectors (Perkus, M. E. *et al.,* In: *Concepts in vaccine development,* Kaufmann, S. H, E., ed., p, 379, 1996; Chakrabarti, S. *et al., Nature* 320:535, 1986; Hu, S. L. *et al., Nature* 320:537, 1986; Kieny, M.-P. *et al., AIDS Bio/Technology* 4:790, 1986; Top, F. H. *et al., J. Infect. Dis.* 124:148, 197 1; Chanda, P. K *et al., Virology* 175:535, 1990), particles of viral or synthetic origin *(e.g.,* Kofler, N. *et al., J Immunol. Methods.* 192:25, 1996; Eldridge, J. H. *et al., Sem. Hematol.* 30:16, 1993; Falo, L. D., Jr. *et al., Nature Med.* 7:649, 1995), adjuvants (Warren, H. S., Vogel, F. R., and Chedid, L. A. *Annul. Rev. Immunol.* 4:369,1986; Gupta, R. K. *et al., Vaccine* 11:293, 1993), liposomes (Reddy, R. *et al., J Immunol.* 148:1585, 1992; Rock, K. L., *Immunol. Today* 17:131, 1996), or, naked or particle absorbed cDNA (Ulmer, J. B. *et al., Science* 259:1745, 1993; Robinson, H. L., Hunt, L. A., and Webster, R. G., *Vaccine* 11:957, 1993; Shiver, J. W. *et al.,* In: *Concepts in vaccine development,* Kaufmann, S. H. E., ed., p. 423, 1996; Cease, K. B., and Berzofsky, J. A., *Annul, Rev. Immunol.* 12:923, 1994 and Eldridge, J. H. *et al., Sem. Hematol.* 30:16, 1993). Toxin-targeted delivery technologies,

also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) can also be used.

Immunomodulatory preparations of the invention include nucleic acid-mediated modalities. DNA or RNA encoding one or more of the peptides of the invention can also be administered to a patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,827,516; 5,880,103 and in more detail below. Examples of nucleic acid -based delivery technologies include "naked DNA", facilitated (bupivicaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or microparticle delivery (U.S. Patent No.5,783,567).

For therapeutic or prophylactic immunization purposes, the peptides of the invention can also be expressed by viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowl pox. As an example of this approach, vaccinia virus is used as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into a host bearing a tumor, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host $CD8^+$ T lymphocyte response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, *U.S.* Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover. *et al.*, *Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g. adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella*

typhi vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein.

Furthermore, immunomodulatory preparations in accordance with the invention encompass compositions comprising one or more of the claimed peptides. A peptide can be present in an immunomodulatory preparation individually. Alternatively, the peptide can exist as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides. Polymers have the advantage of increased immunological reaction and, where different peptide epitopes are used to make up the polymer, the additional ability to induce antibodies and/or $CD8^+$ T lymphocytes that react with different antigenic determinants of the pathogenic organism or tumor-related peptide targeted for an immune response. The composition can be a naturally occurring region of an antigen or can be prepared, *e.g.*, recombinantly or by chemical synthesis.

Carriers that can be used with immunomodulatory preparations of the invention are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly *L*-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like, The immunomodulatory preparations can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, preferably phosphate buffered saline. The immunomodulatory preparations also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are examples of materials well known in the art. Additionally, as disclosed herein, $CD8^+$ T

lymphocyte responses can be primed by conjugating peptides of the invention to lipids, such as tripalrnitoyl-S-glycerylcysteinlyseryl-serine (P_3CSS).

Upon immunization with an peptide composition in accordance with the invention, via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, the immune system of the host responds to the immunomodulatory preparation by producing large amounts of $CD8^+$ T lymphocytes specific for the desired antigen. Consequently, the host becomes at least partially immune to later infection, or at least partially resistant to developing an ongoing chronic infection, or derives at least some therapeutic benefit when the antigen was tumor-associated.

An immunomodulatory preparation of the invention can also include antigen-presenting cells (APC), such as dendritic cells (DC), as a vehicle to present peptides of the invention. Immunomodulatory preparation compositions can be created *in vitro*, following dendritic cell mobilization and harvesting, whereby loading of dendritic cells occurs *in vitro*. For example, dendritic cells are transfected, *e.g.*, with a minigene in accordance with the invention, or are pulsed with peptides. The dendritic cell can then be administered to a patient to elicit immune responses *in vivo*.

Immunomodulatory preparation compositions, either nucleic acid - or peptide-based, can also be administered *in vivo* in combination with dendritic cell mobilization whereby loading of dendritic cells occurs *in vivo*.

Antigenic peptides are used to elicit a $CD8^+$ T lymphocytes response *ex vivo*, as well. The resulting $CD8^+$ T

lymphocytes can be used to treat tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a therapeutic immunomodulatory peptide or nucleic acid in accordance with the invention. *Ex vivo* $CD8^+$ T lymphocyte response to a particular tumor-associated antigen are induced by incubating in tissue culture the patient's, or genetically compatible, $CD8^+$ T lymphocyte precursor cells together with a source of antigen-presenting cells, such as dendritic cells, and the appropriate immunogenic peptide. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded into effector cells, the cells are infused back into the patient, where they will destroy ($CD8^+$ T lymphocytes) their specific target cell (an infected cell or a tumor cell). Transfected dendritic cells may also be used as antigen presenting cells.

The immunomodulatory compositions of the invention can also be used in combination with other treatments used for cancer, including use in combination with immune adjuvants such as IL-2, IL-12, GM-CSF, and the like.

Preferably, the following principles are utilized when selecting an array of epitopes for inclusion in a polyepitope composition for use in an immunomodulatory preparation, or for selecting discrete epitopes to be included in an immunomodulatory preparation and/or to be encoded by nucleic acids such as a minigene. Exemplary epitopes that may be utilized in an immunomodulatory preparation to treat or prevent cancer include YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID

NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8). It is preferred that each of the following principles are balanced in order to make the selection. The multiple epitopes to be incorporated in a given immunomodulatory composition can be, but need not be, contiguous in sequence in the native antigen from which the epitopes are derived.

1.) Epitopes are selected which, upon administration, mimic immune responses that have been observed to be correlated with tumor clearance. For HLA Class I this includes 3-4 epitopes that come from at least one TAA. Epitopes from one TAA may be used in combination with epitopes from, one or more additional TAAs to produce an immunomodulatory preparation that targets tumors with varying expression patterns of frequently-expressed TAAs.

2.) When selecting epitopes from cancer-related antigens it is often useful to select analogs because the patient may have developed tolerance to the native epitope. When selecting epitopes for infectious disease-related antigens it is preferable to select either native or analog epitopes.

3.) Of particular relevance are epitopes referred to as "nested epitopes." Nested epitopes occur where at least two epitopes overlap in a given peptide sequence. A nested peptide sequence can comprise both HLA class I and HLA class II epitopes. When providing nested epitopes, a general objective is to provide the greatest number of epitopes per sequence. Thus, an aspect is to avoid providing a peptide that is any longer than the amino terminus

of the amino terminal epitope and the carboxyl terminus of the carboxyl terminal epitope in the peptide. When providing a multi-epitope sequence, such as a sequence comprising nested epitopes, it is generally important to screen the sequence in order to insure that it does not have pathological or other deleterious biological properties.

A number of different approaches are available which allow simultaneous delivery of multiple epitopes. Nucleic acids encoding the peptides of the invention are a particularly useful embodiment of the invention. Epitopes for inclusion in a minigene are preferably selected according to the guidelines set forth in the previous section. A preferred means of administering nucleic acids encoding the peptides of the invention uses minigene constructs encoding a peptide comprising one or multiple epitopes of the invention.

The immunogenicity of a multi-epitope minigene can be tested in transgenic mice to evaluate the magnitude of $CD8^+$ T lymphocyte induction responses against the epitopes tested. Further, the immunogenicity of nucleic acid - encoded epitopes in vivo can be correlated with the *in vitro* responses of specific $CD8^+$ T lymphocyte lines against target cells transfected with the nucleic acid plasmid. Thus, these experiments can show that the minigene serves to both: 1) generate a $CD8^+$ T lymphocyte response and 2) that the induced $CD8^+$ T lymphocytes recognized cells expressing the encoded epitopes. Persons skilled in the art can also refer to U.S. Patent Nos: 5,827,516; 5,880,103; 6,013,258; 6,183,746 and 5,783,567 and U.S.

Patent Application Nos: 09/321,346; 09/872,836; 60/262,219 (incorporated herein by reference) which describe the use of minigenes, nucleic acid encoded immunomodulatory preparations and delivery of such agents for therapeutic use.

For example, to create a nucleic acid sequence encoding the selected epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes may be reverse translated. A human codon usage table can be used to guide the codon choice for each amino acid. These epitope-encoding nucleic acid sequences may be directly adjoined, so that when translated, a continuous polypeptide sequence is created. To optimize expression and/or immunogenicity, additional elements can be incorporated into the minigene design. Examples of amino acid sequences that can be reverse translated and included in the minigene sequence include: HLA class I epitopes, HLA class II epitopes, a ubiquitination signal sequence, and/or an endoplasmic reticulum targeting signal. In addition, HLA presentation of $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte epitopes may be improved by including synthetic (eg. Poly-alanine) or naturally-occurring flanking sequences adjacent to the $CD8^+$ T lymphocyte or $CD4^+$ T lymphocyte epitopes; these larger peptides comprising the epitope(s) are within the scope of the invention.

The peptides of the present invention and pharmaceutical and immunomodulatory compositions of the invention are typically used therapeutically to treat cancer. Immunomodulatory compositions containing the peptides of the invention are typically administered to a cancer patient who has a malignancy associated with

expression of one or more tumor-associated antigens. Alternatively, immunomodulatory compositions can be administered to an individual susceptible to, or otherwise at risk for developing a particular type of cancer, *e.g.*, breast cancer.

In therapeutic applications, peptide and/or nucleic acid compositions are administered to a patient in an amount sufficient to elicit an effective $CD8^+$ T lymphocyte response to the tumor antigen and to cure or at least partially arrest or slow symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular composition administered, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

As noted above, peptides comprising $CD8^+$ T lymphocyte epitopes of the invention induce immune responses when presented by HLA molecules and contacted with a $CD8^+$ T lymphocyte specific for an epitope comprised by the peptide. The manner in which the peptide is contacted with the $CD8^+$ T lymphocyte is not critical to the invention. For instance, the peptide can be contacted with the $CD8^+$ T lymphocyte either in vivo or *in vitro*. If the contacting occurs *in vivo*, the peptide itself can be administered to the patient, or other vehicles, *e.g.*, nucleic acid vectors encoding one or more peptides, viral vectors encoding the peptide(s), liposomes and the like, can be used, as described herein.

When the peptide is contacted *in vitro*, the vaccinating agent can comprise a population of cells, *e.g.*, peptide-pulsed dendritic cells, or TAA-specific $CD8^+$ T lymphocytes, which have been induced by pulsing antigen-presenting cells *in vitro* with the peptide. Such a cell population is subsequently administered to a patient in a therapeutically effective dose.

For pharmaceutical compositions, the immurogenic peptides of the invention, or nucleic acid encoding them, are generally administered to an individual already diagnosed with cancer. The peptides or nucleic acid encoding them can be administered individually or as fusions of one or more peptide sequences. For therapeutic use, administration should generally begin at the first diagnosis of cancer. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. The embodiment of the immunomodulatory composition (i.e., including, but not limited to embodiments such as peptide cocktails, polyepitope polypeptides, minigenes, or TAA-specific $CD8^+$ T lymphocytes) delivered to the patient may vary according to the stage of the disease. For example, a immunomodulatory preparation comprising TAA-specific $CD8^+$ T lymphocytes may be more efficacious in killing tumor cells in patients with advanced disease than alternative embodiments.

The immunomodulatory compositions of the invention may also be used therapeutically in combination with treatments such as surgery. An example is a situation in which a patient has undergone surgery to remove a primary tumor and the immunomodulatory preparation is then used to slow or prevent recurrence and/or metastasis.

Where susceptible individuals, *e.g.*, individuals who may be diagnosed as being genetically pre-disposed to developing a particular type of tumor, are identified prior to diagnosis of cancer, the composition can be targeted to them, thus minimizing the need for administration to a larger population.

The dosage for an initial therapeutic immunization generally occurs in a unit dosage range where the value is about 1, 5, 50, or 500 μg. Dosage values for a human typically range from about 50 μg to about 500 μg per 70 kilogram patient. Boosting dosages of between about 50 μg to about 100 μg of nucleic acid pursuant to a boosting regimen over weeks to months may be administered depending upon the patient's response and condition as determined by measuring the specific activity of $CD8^+$ T lymphocyte obtained from the patient's blood.

Administration should continue until at least clinical symptoms or laboratory tests indicate that the tumor has been eliminated or that the tumor cell burden has been substantially reduced and for a period thereafter. The dosages, routes of administration, and dose schedules are adjusted in accordance with methodologies known in the art.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral, intrathecal, or local administration. Preferably, the pharmaceutical compositions are administered parentally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A

variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH-adjusting and buffering agents, tonicity adjusting agents, wetting agents, preservatives, and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monalaurate, triethanolamine oleate, *etc.*

The concentration of peptides of the invention in the pharmaceutical formulations can vary widely, *i. e.*, from less than about 0. 1 %, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, *in* accordance with the particular mode of administration selected.

A human unit dose form of the peptide composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, preferably an aqueous carrier, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans (*see, e.g.*, Remington's Pharmaceutical Sciences, 17" Edition, A. Gennaro, Editor, Mack Publishing Co., Easton, Pennsylvania, 1985).

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or to target selectively to infected cells, as well as to increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations, the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the peptide compositions. Liposomes for use in accordance with the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. Ile selection of lipids is generally guided by consideration *25 of, e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.* , Szoka, et *al.*, *Ann. Rev. Biophys. Bioeng*. 9:467 (1980), and U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

For targeting cells of the immune system, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered

intravenously, locally, topically, *etc.* in a dose which varies according to, *inter alia*, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcurn, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1 %-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1 %-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In the cancer setting there are several findings that indicate that immune responses can impact neoplastic growth:

First, the demonstration in many different animal models, that anti-tumor T cells, restricted by MHC class I, can prevent or treat tumors.

Second, encouraging results have come from immunotherapy trials.

Third, observations made in the course of natural disease correlated the type and composition of T cell infiltrate within tumors with positive clinical outcomes (Coulie PG, et al. Antitumor immunity at work in a melanoma patient In Advances in Cancer Research, 213-242, 1999).

Finally, tumors commonly have the ability to mutate, thereby changing their immunological recognition. For example, the presence of monospecific $CD8^+$ T lymphocytes was also correlated with control of tumor growth, until antigen loss emerged (Riker A, et al., Immune selection after antigen-specific immunotherapy of melanoma Surgery, Aug: 126(2):112-20, 1999; Marchand M, et al., Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1 Int. J Cancer 80(2):219-3 0, Jan. 18, 1999). Similarly, loss of beta 2 microglobulin was detected in 5113 lines established from melanoma patients after receiving immunotherapy at the NCI (Restifo NP, el al., Loss of functional Beta-2-microglobulin in metastatic melanomas from five patients receiving immunotherapy Journal of the National Cancer Institute, Vol. 88 (2), 100-108, Jan. 1996). It has long been recognized that HLA class I is frequently altered in various tumor types. This has

led to a hypothesis that this phenomenon might reflect immune pressure exerted on the tumor by means of class I restricted $CD8^+$ T lymphocytes. The extent and degree of alteration in HLA class I expression appears to be reflective of past immune pressures, and may also have prognostic value (van Duinen SG, et al., Level of HLA antigens in locoregional metastases and clinical course of the disease in patients with melanoma Cancer Research 48, 1019-1025, Feb. 1988; Moller P, et al., Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma Cancer Research 51, 729-736, Jan. 1991). Taken together, these observations provide a rationale for immunotherapy of cancer and infectious disease, and suggest that effective strategies need to account for the complex series of pathological changes associated with disease.

Therapeutic Use in Cancer Patients

Evaluation of immunomodulatory compositions are performed to validate the efficacy of the $CD8^+$ T lymphocyte-$CD4^+$ T lymphocyte peptide compositions in cancer patients. The main objectives of the trials are to determine an effective dose and regimen for inducing $CD8^+$ T lymphocytes in cancer patients, to establish the safety of inducing a $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte response in these patients, and to see to what extent activation of $CD8^+$ T lymphocytes improves the clinical picture of cancer patients, as manifested by a reduction in tumor cell numbers. Such a study is designed, for example, as follows:

The studies are performed in multiple centers. The trial design is an open-label, uncontrolled, dose escalation protocol wherein the immunomodulatory

composition is administered as a single peptide dose followed six weeks later by a single booster shot of the same dose. The dosages are 500, 500 and 5,000 micrograms per injection. Drug-associated adverse effects (severity and reversibility) are recorded.

There are three patient groupings. The first group is injected with 50 micrograms of the immunomodulatory composition and the second and third groups with 500 and 5,000 micrograms of immunomodulatory peptide composition, respectively. The patients within each group range in age from 21-65, include both males and females (unless the tumor is sex-specific, e.g., breast or prostate cancer), and represent diverse ethnic backgrounds.

Induction of $CD8^+$ T lymphocyte Responses Using a Prime Boost Protocol

A prime boost protocol similar in its underlying principle to that used to evaluate the efficacy of a nucleic acid immunomodulatory preparation in transgenic mice, which was described in above, may also be used for the administration of the immunomodulatory preparation to humans. Such a therapeutic regimen may include an initial administration of, for example, naked or microencapsulated nucleic acid followed by a boost using recombinant virus encoding the immunomodulatory preparation, or recombinant protein/polypeptide or a peptide mixture administered in an adjuvant.

For example, the initial immunization may be performed using an expression vector, such as that described above, in the form of naked or microencapsulated nucleic acid administered IM (or SC or ID) in the amounts of 0.5-500 ug at multiple sites. The nucleic acid (0.1 to 500 ug) can also be administered using a gene gun. Following an incubation period of 3-4 weeks, a booster dose is then administered. The booster can be recombinant fowlpox virus

administered at a dose of $5x10^7$ to $5x10^9$ pfu. An alternative recombinant virus, such as an MVA, canarypox, adenovirus, or adeno-associated virus, can also be used for the booster, or the polyepitope protein, a mixture of the peptides or microencapsulated plasmid DNA encoding the epitopes can be administered. For evaluation of immunomodulatory efficacy, patient blood samples will be obtained before immunization as well as at intervals following administration of the initial immunomodulatory preparation and booster doses. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient sedimentation, aliquoted in freezing media and stored frozen. Samples are assayed for $CD8^+$ T lymphocyte and $CD4^+$ T lymphocyte activity.

Analysis of the results will indicate that a magnitude of response sufficient to achieve protective immunity against cancer is generated.

Adjunct Therapy to Surgery

The peptides of the invention can be used to activate $CD8^+$ memory T cells specific for CEA by *in vitro* methods known to those in the art. After a patient undergoes surgery to remove a tumor, such CEA-specific $CD8^+$ memory T cells may be administered to establish tumor-specific, long-lasting immunity against tumor rechallenge.

Reagents that Bind to Peptide-Class I MHC Complexes

Reagents that bind to peptide-class I MHC complexes can be made, for example, by screening a phage display library in which the phage particles contain nucleic acid sequences encoding antibody fragments such as Fab or single

chain Fv (scFv) fragments. Such libraries can be screened by testing for the presence of and isolating phage particles with the ability to bind to the peptides of the invention (e.g., YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), bound to a class I MHC molecule of interest. For example, phage display technology has been used to identify antibodies specific for defined HLA-A1 peptide complexes associated with melanoma [Chames et al. (2000), PNAS 97:7969-7974]. Alternatively, polyclonal antibodies or mAb can be screened for their ability to bind to peptide-class I MHC complexes of interest.

The above antibody-based reagents can be used, for example, in diagnosing cancer. For example, binding to a test cell of a Fab, scFv, or an antibody (e.g., a mAb) specific for a HLA-A2 molecule bound to a CEA peptide of the invention would indicate that the test cell is a cancer cell. Examples of detection agents used to detect binding of antibodies or antibody fragments include, without limitation, enzymes, radiolabels, luminescent compounds, and fluorescing compounds that elicit a detectable and measurable signal when the antibody complexes with the CEA naturally processed peptide. Examples of detectors include, without limitation, spectrophotometers, colorimeters, fluorometers, luminometers and biacore machines.

Therapeutic agents for treating cancer can be made, for example, by linking one of the above reagents with a therapeutic (e.g., cytotoxic) atom or molecule. An appropriate therapeutic agent, after administration (by any

of the methods disclosed herein) to a subject with the relevant cancer, binds to the cancer cells. The therapeutic atom or molecule can then kill the cancer cell. Alternatively, the therapeutic agent is internalized by the cancer cell and then the therapeutic atom or molecule kills the cancer cell. The linkage between the reagent and therapeutic atom or molecule can be a covalent one or a relatively weak non-covalent one such that the complex dissociates after binding to the cancer cell surface. Examples of therapeutic atoms and molecules include chemotherapeutic compounds, radioisotopes, and toxins, e.g., ricin or diptheria toxin, or toxic fragments of such toxins.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of identifying a class I MHC-binding fragment of a polypeptide, the method comprising:

(a) isolating from the tissue/cell line a class I MHC molecule bound to a peptide, wherein the peptide is a class I MHC-binding fragment of the polypeptide;

(b) eluting the peptide from the class I MHC molecule; and

(c) identifying the peptide as a fragment of the polypeptide

2. The method of claim 1, wherein the polypeptide has the sequence of a tumor antigen.

3. The method of claim 1, wherein the tissue is a tumor.

4. The method of claim 1, wherein the cell line is a tumor cell line.

5. The method of claim 1, wherein the mammal is a human.

6. The method of claim 1, wherein the cell line is any mammalian cell that expresses class I MHC molecules on its surface.

7. The method of claim 1, wherein the class I MHC molecule is selected from the group consisting of a HLA-A molecule, a HLA-B molecule, and a HLA-C molecule.

8. The method of claim 1, wherein the class I MHC molecule is encoded by a gene selected from the group consisting of HLA-A1, HLA-A2, HLA-A3, HLA-A11, HLA-A24, HLA-B7, HLA-B8, HLA-B35, HLA-B44, HLA-B62 and HLA-B63.

9. The method of claim 1, wherein the class I MHC molecule is encoded by a gene selected from the group

consisting of A*0101, A*0201, A*0301, A*1101, A*2402, B*0702, B*0801, B*1502, B*3501, B*4401, B*5301 and B*5401.

10. A peptide fewer than 100 amino acids in length, the peptide comprising at least one isolated, naturally processed epitope consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8), wherein the peptide constitutes less than 70% of a naturally occurring CEA protein.

11. The peptide of claim 10, comprising at least two isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

12. The peptide of claim 10, comprising at least three isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

13. The peptide of claim 12, where the three isolated, naturally processed epitopes comprise the sequences ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI

(SEQ ID NO:8).

14. A peptide fewer than 100 amino acids in length, the peptide consisting of at least one isolated, naturally processed epitope consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8), wherein the peptide constitutes less than 70% of a naturally occurring CEA protein.

15. The peptide of claim 14, consisting of at least two isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

16. The peptide of claim 14, consisting of at least three isolated, naturally processed epitopes consisting of a sequence selecting from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL(SEQ ID NO:4), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI (SEQ ID NO:8).

17. The peptide of claim 16, where the three isolated, naturally processed epitopes consist of the sequences ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), GVLVGVALI

(SEQ ID NO:8).

18. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNNQSL (SEQ ID NO:1).

19. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNDQSL (SEQ ID NO:2).

20. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLWWVNGQSL (SEQ ID NO:3).

21. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence YLSGANLNL (SEQ ID NO:4).

22. An isolated peptide fewer than 22 amino acid residues in length, comprising a sequence ATVGIMIGVLVGVALI (SEQ ID NO:5).

23. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence ATVGIMIGV (SEQ ID NO:6).

24. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence IMIGVLVGV (SEQ ID NO:7).

25. An isolated peptide fewer than 15 amino acid residues in length, comprising a sequence GVLVGVALI (SEQ ID NO:8).

26. The peptide library of isolated peptide fewer than 100 amino acid in length comprising the sequences YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8).

27. An altered peptide ligand (APL), the amino acid sequence of which is identical, except for 1-6 amino acid substitutions, to a fragment of carcinoembryonic antigen (CEA), the fragment being fewer than 20 amino acids residues in length and comprising a sequence selected from the group consisting of YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8), optionally with additional CEA sequence on one or both ends, wherein no more than 30% of the amino acid residues of the fragment are substituted with different amino acid residues in the APL, and wherein the APL binds to a class I MHC molecule.

28. A process for making an APL, the process comprising:

a. carrying out the method of claim 1, and
b. synthesizing an APL consisting of a sequence which is identical to that of the peptide, except having amino acid substitutions at 1, 2, 3, 4, 5, or 6 positions in the peptide.

29. The process of claim 21, wherein the polypeptide is CEA.

30. A method of activating T cell reactivity in a mammal, the method comprising:

a. providing (i) a peptide, the sequence of which consists of the sequence of a naturally processed fragment of CEA, wherein the peptide binds to a class I MHC molecule of the mammal and elicits a $CD8^+$ T cell response, or (ii) a nucleic acid encoding a polypeptide selected from the group consisting of (1) the peptide, (2) the peptide plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and

b. administering the peptide or nucleic acid to the mammal.

31. The method of claim 23, wherein the peptide is YLWWVNNQSL (SEQ ID NO:1).

32. The method of claim 23, wherein the peptide is YLWWVNDQSL (SEQ ID NO:2).

33. The method of claim 23, wherein the peptide is YLWWVNGQSL (SEQ ID NO:3).

34. The method of claim 23, wherein the peptide is YLSGANLNL (SEQ ID NO:4).

35. The method of claim 23, wherein the peptide is ATVGIMIGVLVGVALI (SEQ ID NO:5).

36. The method of claim 23, wherein the peptide is ATVGIMIGV (SEQ ID NO:6).

37. The method of claim 23, wherein the peptide is IMIGVLVGV (SEQ ID NO:7).

38. The method of claim 23, wherein the peptide is GVLVGVALI (SEQ ID NO:8).

39. A method of altering a T cell response in a mammal, the method comprising:

a. providing (i) an APL having a sequence identical, except for amino acid substitutions at 1-6 positions, to the sequence of a naturally-processed fragment of CEA, wherein the APL binds to a class I MHC molecule of the mammal, or (ii) a nucleic acid encoding a polypeptide selected from the group consisting of (1) the APL, (2) the APL plus an amino terminal methionine residue, and (3) either (1) or (2) linked to a trafficking sequence; and

b. administering the APL or nucleic acid to the mammal.

33. The method of claim 1, further comprising:

(a) providing $CD8^+$ lymphocytes from a mammal having a condition suspected of being associated with presentation of the peptide by the class I MHC molecule, wherein the tumor cells of the mammal bear the class I MHC molecule;

(b) providing a population of tumor cells that bear the class I MHC molecule with the peptide bound thereto;

(c) contacting the population of tumor cells of (b) with the $CD8^+$ lymphocytes of (a); and

(d) determining whether the $CD8^+$ lymphocytes recognize the class I MHC-bound peptide, as an indication that presentation of the peptide to

$CD8^+$ T lymphocytes is associated with the condition.

34. The method of claim 33, wherein said presentation is associated with a pathological response of $CD8^+$ T lymphocytes.

35. The method of claim 33, wherein said presentation is associated with a protective response of $CD8^+$ T lymphocytes.

36. A method of diagnosis comprising:

(a) isolating an individual suspected of having or being susceptible to cancer;

(b) providing a $CD8^+$ lymphocyte from the individual;

(c) providing an APC which bears on its surface a class I MHC molecule of an allele identical to one expressed by said individual, wherein the class I MHC molecule is bound to a CEA peptide;

(d) contacting the APC with the $CD8^+$ lymphocyte; and

(e) determining whether the $CD8^+$ lymphocyte recognizes the class I MHC-bound peptide, as an indication that the individual has or is susceptible to cancer,

wherein the peptide comprises an amino acid sequence selected from the group consisting of: YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8).

37. A method of treating cancer, the method comprising:

(a) isolating a subject suspected of having or being susceptible to cancer; and

(b) administering the peptide of claim 10 to the subject.

38. A method of identifying a reagent for diagnosing cancer, the method comprising:

(a) providing a test reagent selected from the group consisting of a Fab fragment, a monoclonal antibody (mAb), and a single chain Fv (scFv) fragment;

(b) providing a complex comprising a class I MHC molecule bound to a peptide selected from the group consisting of YLWWVNNQSL (SEQ ID NO:1), YLWWVNDQSL (SEQ ID NO:2), YLWWVNGQSL (SEQ ID NO:3), YLSGANLNL (SEQ ID NO:4), ATVGIMIGVLVGVALI (SEQ ID NO:5), ATVGIMIGV (SEQ ID NO:6), IMIGVLVGV (SEQ ID NO:7), or GVLVGVALI (SEQ ID NO:8); and

(c) testing whether the test reagent binds to the complex.

39. The method of claim 38, wherein the class I MHC molecule is a HLA-A2 molecule encoded by a A*0201 gene.

40. A method of diagnosis, the method comprising:

(a) providing a test cell from a mammalian subject;

(b) providing a reagent that binds to a CEA peptide fragment bound to a class I MHC molecule;

(c) contacting the test cell with the reagent; and

(d) detecting binding of the reagent to the test cell as an indication that the test cell is a cancer cell.

41. A method of cancer treatment, the method comprising:

(a) isolating a subject suspected of having or being susceptible to cancer;

(b) providing a composition comprising a reagent selected from the group consisting of a Fab fragment, a mAb, and a scFv fragment, wherein the reagent recognizes a naturally processed CEA peptide bound to a MHC class I molecule, the reagent being linked to an agent selected from the group consisting of a chemotherapeutic compound, a radioactive isotope and a toxin; and

(c) administering the composition to the subject; wherein the cancer is characterized by expression of CEA and the administration results in an amelioration of one or more symptoms of the cancer.

42. A method of identifying a class I MHC-binding fragment of a tumor antigen, the method comprising:

(a) providing a mammalian tumour tissue or tumour cell line comprising a class I MHC molecule and the tumour antigen;

(b) isolating from the tissue/cell line the class I MHC molecule bound to a peptide, wherein the

peptide is a class I MHC binding fragment of the tumour antigen;

(c) eluting the peptide from the class I MHC molecule; and

(d) identifying the amino acid sequence of the peptide.

43. The method of claim 42, further comprising:

(e) providing $CD8^+$ lymphocytes from a mammal having a cancer suspected of being associated with presentation of the peptide by the class I MHC molecule, wherein the tissue/cell lines of the mammal bear the class I MHC molecule;

(f) providing a population of tumour cell line that bear the class I MHC molecule with the peptide bound thereto;

(g) contacting the population of tumour cell line of (f) with the $CD8^+$ lymphocytes of (e); and

(h) determining whether the $CD8^+$ lymphocytes recognize the class I MHC bound peptide, as an indication that presentation of the peptide to $CD8^+$ lymphocytes is associated with the cancer.

44. An isolated nucleic acid comprising a nucleotide sequence encoding the peptide of claim 10 or a peptide of fewer than 20 amino acids in length and comprising the sequence of SEQ ID NOS:1-8.

45. A vector comprising the nucleic acid of claim 44.

46. The vector of claim 45, wherein the nucleotide sequence is operatively linked to a transcriptional regulatory element.

47. A cell comprising the vector of claim 45.

48. A cell comprising the vector of claim 46.

49. A method of enhancing an immune response to an antigen in animal comprising

(a) administering an effective amount of an inducing agent to the animal followed by

(b) administering an effective amount of the inducing agent and the antigen to the animal.

50. A method according to claim 49 where the inducing agent is a peptide or nucleic acid encoding a sequence from claim 10 and the antigen is CEA.

51. The peptide encoded by the nucleic acid of claim 44.

52. A polymeric delivery matrix selected from the group consisting of microspheres, hydrogels and polymeric networks and the nucleic acid of claim 44.

53. The polymeric delivery matrix of claim 52 where the matrix comprises a plurality of microspheres.

54. The microspheres of claim 53, wherein the polymeric matrix consists essentially of a polymer of poly-*co*- glycolic acid (PLGA).

55. A therapeutic composition comprising the nucleic acid of claim 44 and a pharmaceutically acceptable carrier.

56. The therapeutic composition of claim 56, further including an adjuvant.

57. A liposome comprising the nucleic acid of claim 44.

58. A method of eliciting an immune response in a mammal, which method comprises administering the nucleic acid of claim 44 to the mammal.

59. The method of claim 58, wherein the mammal is human.

60. The method of claim 59, wherein the pathogenic agent is CEA and the human suffers from, or is at risk of, cancer.

61. The method of claim 58, wherein the nucleic acid is administered directly to a mucosal tissue of the mammal.

62. The method of claim 58, wherein the nucleic acid is administered subcutaneously or intramuscularly.

63. A method of eliciting an immune response in a mammal, which method comprises administering the microspheres of claim 53 to the mammal

64. A method of treating a subject suspected of having

or being susceptible to cancer, the method comprising isolating the subject and administering the nucleic acid of claim 44 to the subject, wherein the administration results in an amelioration of one or more symptoms of cancer.

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65. The method of claim 64, wherein administering the nucleic acid results in a decrease in a tumour size or activity.

10

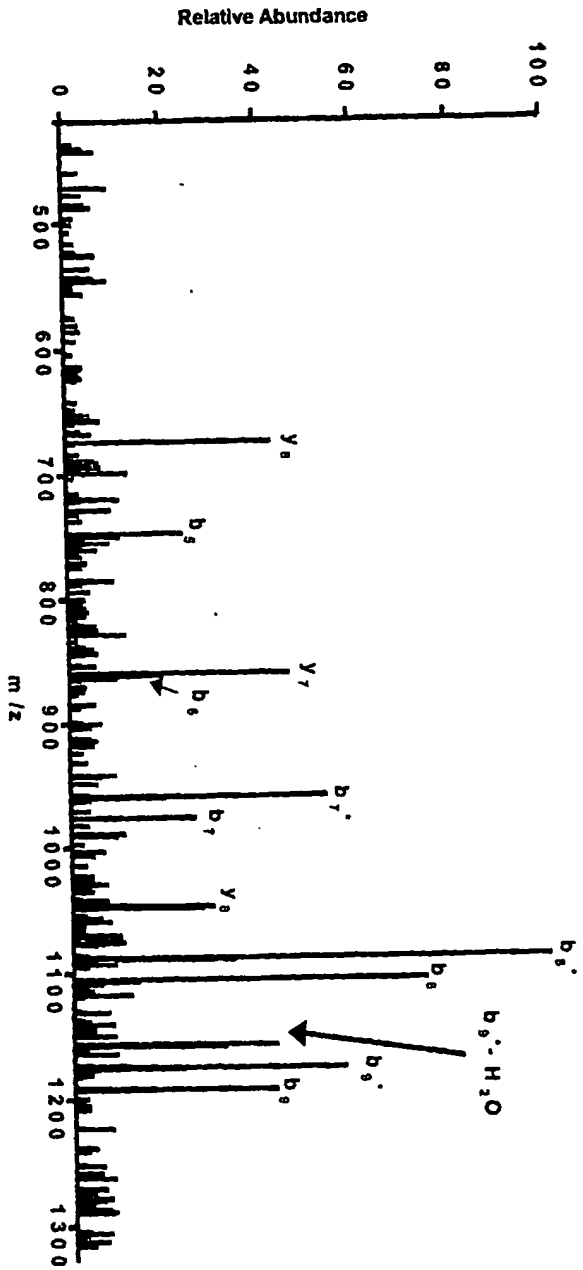
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FIGURE 1

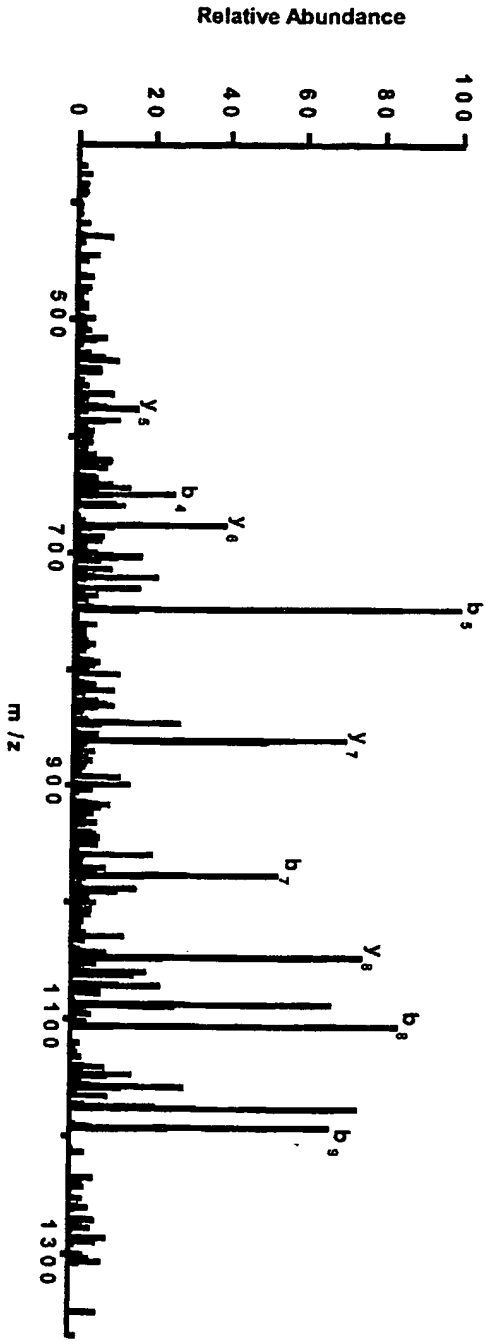
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FIGURE 2

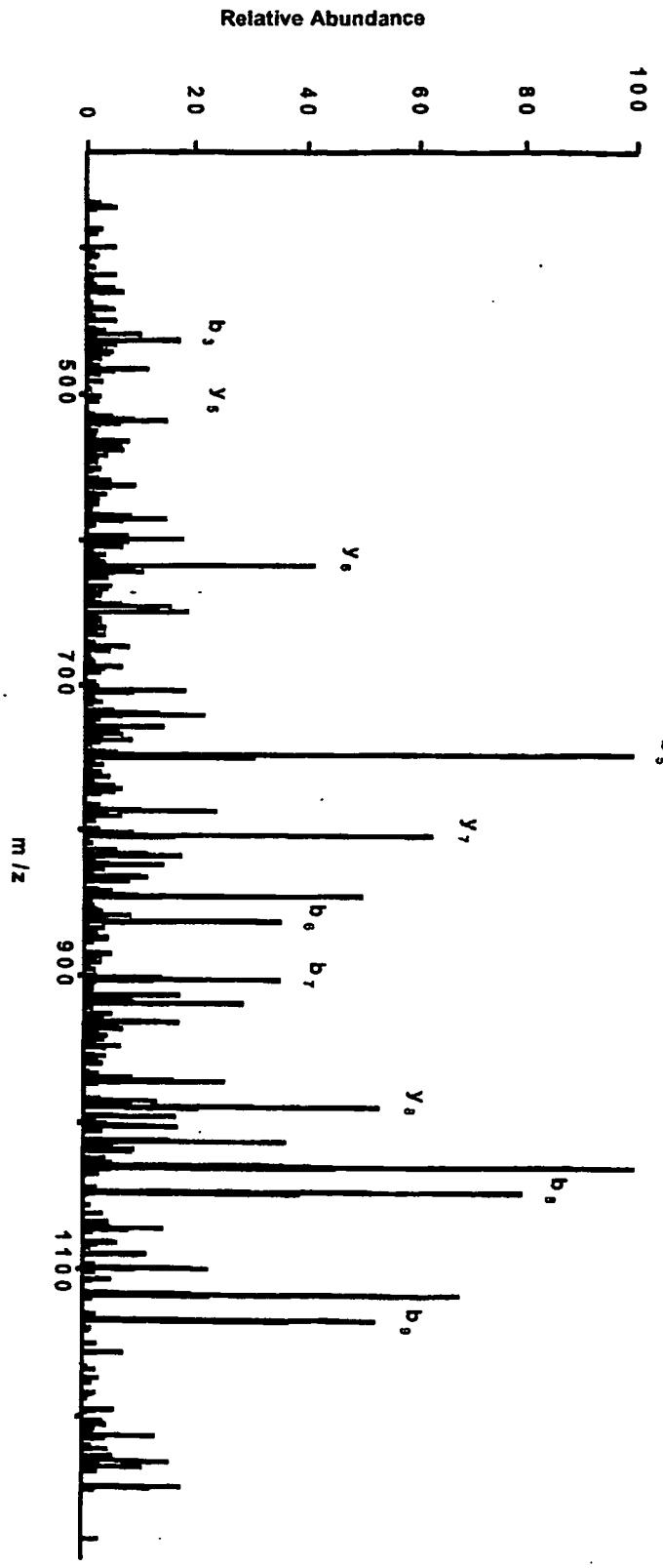
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FIGURE 3

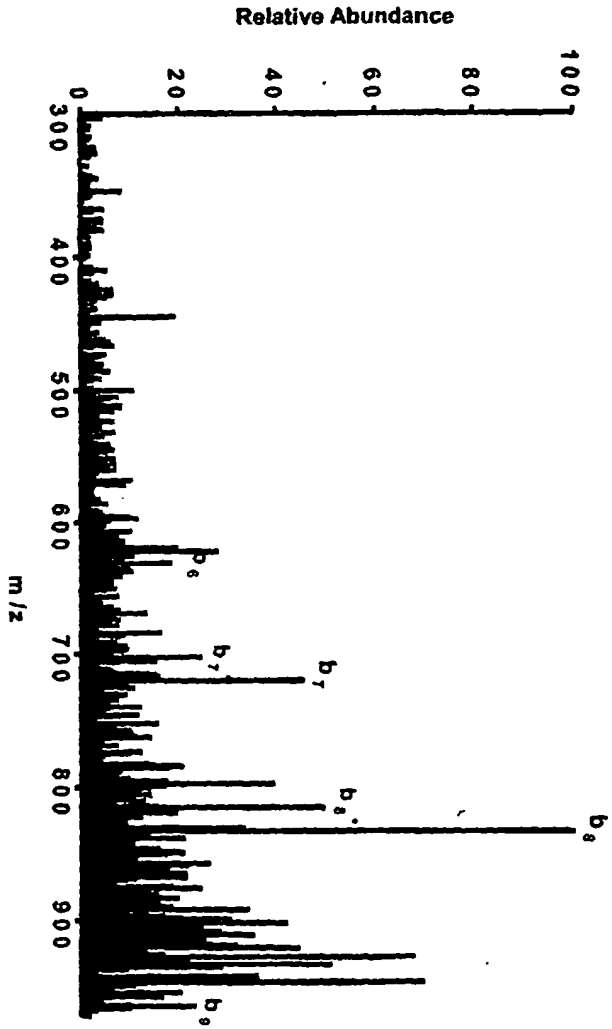
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FIGURE 4

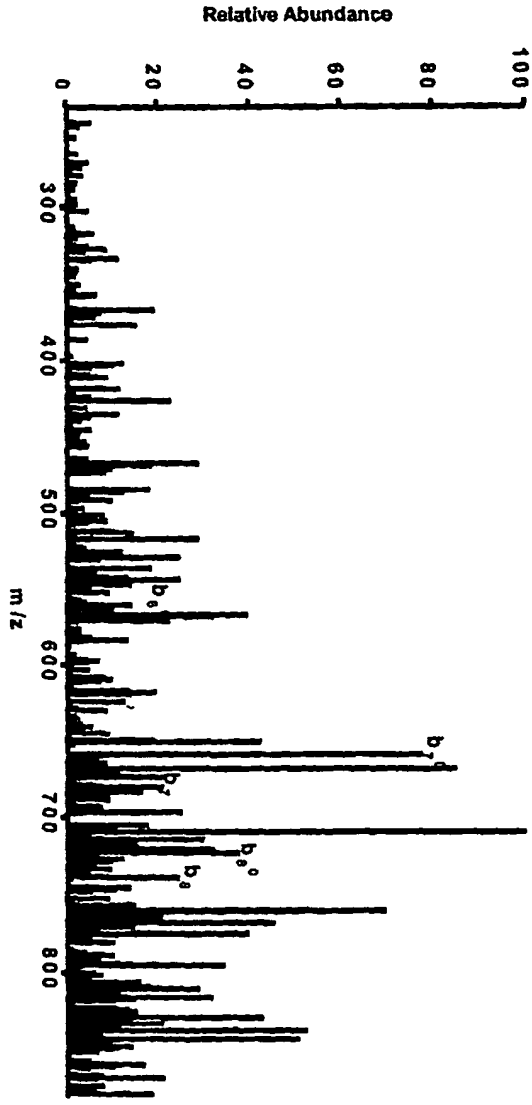
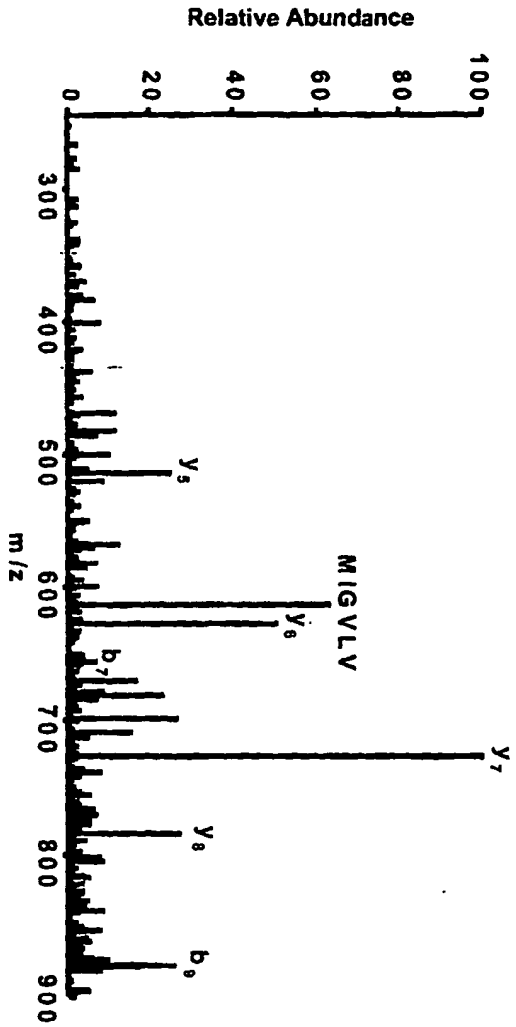


FIGURE 5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700
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m/z
MIGVLV
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y_6
b_7
y_7
y_8
b_9

FIGURE 6

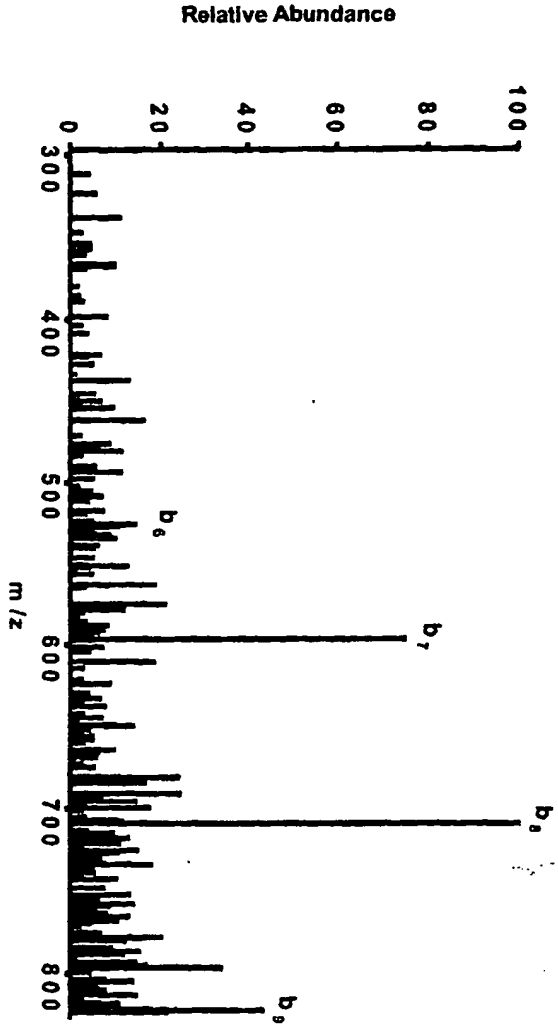
Relative Abundance
0
20
40
60
80
100
300
400
500
600
700
800
m/z
b_6
b_7
b_8
b_9

FIGURE 7

```
MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE VLLLVHNLPQ
HLFGYSWYKG ERVDGNRQII GYVIGTQQAT PGPAYSGREI IYPNASLLIQ NIIQNDTGFY
TLHVIKSDLV NEEATGQFRV YPELPKPSIS SNNSKPVEDK DAVAFTCEPE TQDATYLWWV
NNQSLPVSPR LQLSNGNRTL TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP
TISPLNTSYR SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ NTTYLWWVNN
QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS VDHSDPVILN VLYGPDDPTI
SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL IDGNIQQHTQ ELFISNITEK NSGLYTCQAN
NSASGHSRTT VKTITVSAEL PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS
LPVSPRLQLS NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN GTYACFVSNL
ATGRNNSIVK SITVSASGTS PGLSAGATVG IMIGVLVGVA LI
```

FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/08427

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/04; C07K 7/00
US CL : 530/300, 327,328; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/300, 327,328; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG-Medline, Embase, Cancerlit, Scisearch, Biosis; BRS/EAST-USPatfull, EPO, JPO, Derwint, PGPubs

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 6,319,496 B1 (PANICALI et al.) 20 November 2001 (20.11.01), see entire document, particularly, Table 7 and claims 1-16.	10,14,21,30,34,44-51,55-56,58-62,64-65 ——— 11-13, 15-20, 22-29, 31-33, 35-41, 52-54, 57, 63
X — Y	US 6,001,349 (PANICALI et al.)14 December 1999 (14.12.99), see entire document, especially Table 7 and claims 1-12.	10,14,21,30,34,44-51,55-56,58-62,64-65 ——— 11-13, 15-20, 22-29, 31-33, 35-41, 52-54, 57, 63
X	HUNT. D. F. et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science. March 1992, Vol. 255, 1261-1263, see entire document.	1-9, 42-43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2003 (23.06.2003)	06 AUG 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Anne Marie S. Wehbe Telephone No. 703-308-1235

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INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	RAS. E. et al. Identification of potential HLA-A 0201 Resricted CTL epitopes Derived from the epithelial cell adhesion molecule (Ep-CAM) and the carcinoembryonic antigen (CEA). Human Immunol. 1997, Vol. 53, pages 81-89, see entire document.	10,14,21,30,34,51 --------- 11-13, 15-20, 22-29, 31-33, 35-41, 44-50, 52-64
X	HUANG. A. Y.C. et al. The immunodominant major histocompatibility complex class I restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. PNAS. 1996, Vol. 93, pages 9730-9735, see entire document.	1-9, 42-43